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Primary Colonizing *Betaproteobacteriales* Play a Key Role in the Growth of *Legionella pneumophila* in Biofilms on Surfaces Exposed to Drinking Water Treated by Slow Sand Filtration

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ABSTRACT Slow sand filtration with extensive pretreatment reduces the microbial growth potential of drinking water to a minimum level at four surface water supplies in The Netherlands. The potential of these slow sand filtrates (SSFs) to promote microbial growth in warm tap water installations was assessed by measuring biofilm formation and growth of *Legionella* bacteria on glass and chlorinated polyvinylchloride (CPVC) surfaces exposed to SSFs at 37 ± 2°C in a model system for up to six months. The steady-state biofilm concentration ranged from 230 to 3,980 pg ATP cm^{-2} on glass and 1.4 (±0.3)-times-higher levels on CPVC. These concentrations correlated significantly with the assimilable organic carbon (AOC) concentrations of the warm water (8 to 24 μg acetate-C equivalents [ac-C eq] $liter^{-1}$), which were raised about 2 times by mixing cold and heated (70°C) SSFs. All biofilms supported growth of *Legionella pneumophila* with maximum concentrations ranging from 6×10^2 to 1.5×10^5 CFU cm^{-2}. Biofilms after ≤50 days of exposure were predominated by *Betaproteobacteriales*, mainly *Piscinibacter*, *Caldimonas*, *Methyloversatilis*, and an uncultured *Rhodocyclaceae* bacterium. These rapidly growing primary colonizers most likely served as prey for the host amoebae of *L. pneumophila*. *Alphaproteobacteria*, mostly *Xanthobacteraceae*, e.g., *Bradyrhizobium*, *Pseudorhodoplanes*, and other amoeba-resistant bacteria, accounted for 37.5% of the clones retrieved. A conceptual model based on a quadratic relationship between the *L. pneumophila* colony count and the biofilm concentration under steady-state conditions is used to explain the variations in the *Legionella* CFU pg^{-1} ATP ratios in the biofilms.

IMPORTANCE Proliferation of *L. pneumophila* in premise plumbing poses a public health threat. Extended water treatment using physicochemical and biofiltration processes, including slow sand filtration, at four surface water supplies in The Netherlands reduces the microbial growth potential of the treated water to a minimum level, and the distributed drinking water complies with high quality standards. However, heating of the water in warm tap water installations increases the concentration of easily assimilable organic compounds, thereby promoting biofilm formation and growth of *L. pneumophila*. Prevention of biofilm formation in plumbing systems by maintenance of a disinfectant residual during distribution and/or further natural organic matter (NOM) removal is not feasible in the supplies studied. Temperature management in combination with optimized hydraulics and material selection are therefore essential to prevent growth of *L. pneumophila* in premise plumbing systems. Still, reducing the concentration of biodegradable compounds in drinking water by appropriate water treatment is important for limiting the *Legionella* growth potential.

KEYWORDS AOC, *Legionella pneumophila*, biofilm, heated water, host amoebae, primary colonizers, slow sand filtrate

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The increasing numbers of Legionnaires' disease (LD) cases reported in Europe (1) and the United States (2) demonstrate that exposure to *Legionella pneumophila* is widespread and difficult to control. For the years 2011 to 2014, *L. pneumophila* has been identified as the main cause of drinking water-associated cases of disease and death in the United States (3, 4). The *Legionella* problem emerged in Europe in the early 1980s, and in the Netherlands, cases of LD were mainly observed in hospitals in association with the use of warm tap water and among travelers (5). In 1986, the Health Council of the Netherlands recommended notification of legionellosis cases, as well as temperature (T) management ($T < 25$°C and $T > 60$°C) to prevent growth of *L. pneumophila* in premise plumbing systems (5). Following the large LD outbreak at a flower show in 1999 (6), measures for control of *Legionella* were incorporated in drinking water regulations, and investigations into the relationship between drinking water quality, biofilm formation potential (BFP), and the multiplication of *Legionella pneumophila* bacteria in tap water installations were conducted (7–9). The use of a model system, the boiler biofilm monitor (BBM), representing worst-case conditions (intermittent flow at 37°C), revealed that *L. pneumophila* can multiply at low biofilm concentrations (BfCs) due to its ability to proliferate within protozoan hosts consuming oligotrophic bacteria that grow on drinking water-exposed surfaces (9).

Slow sand filtration, the final treatment process at four surface water supplies (SWSs I to IV) in the Netherlands, is used for the removal of suspended material, microorganisms, and biodegradable compounds (10). Assessment of the microbial growth potential (MGP) of the slow sand filtrates (SSFs) of these supplies with the assimilable organic carbon (AOC) test, based on simultaneous growth of *Pseudomonas fluorescens* strain P17 and *Spirillum* sp. strain NOX, and the biomass production potential (BPP) test, based on growth of the indigenous bacteria, revealed that the specific MGP (AOC/total organic carbon [TOC] and BPP/TOC) approached the limits achievable by biological filtration at temperatures of ≤20°C (11). The finished water is distributed without disinfectant, and the heterotrophic plate count (HPC) of drinking water in the connected distribution systems is low (annual geometric mean, <10 CFU ml^{-1}), as are coliform counts (<1 CFU 100 ml^{-1}). Hence, regrowth in the distribution system is strictly limited, although some growth of aeromonads may occur (11). However, *L. pneumophila* had been observed in warm tap water installations of hospitals supplied with drinking water treated by slow sand filtration (12–14). Therefore, the BBM system was used to assess the potential of the SSFs to support growth of *L. pneumophila* in warm tap water installations. The objectives of the study were to (i) assess the effects of the concentrations of AOC and TOC in the SSFs on biofilm formation at 37°C, (ii) measure the growth of *L. pneumophila* in these biofilms, (iii) identify the predominating biofilm bacteria, and (iv) analyze the growth of *L. pneumophila* in relation to biofilm concentration and composition.

RESULTS

Study setup. The study included finished water of four SWSs applying slow sand filtration with different pretreatments (Fig. S1 in the supplemental material). The MGP of the SSFs was quantified with the AOC test in water samples heated to 60°C for 30 min and after heating the water to 70°C for 6 h. BBMs, operating under controlled hydraulic conditions with water at 37°C, obtained by mixing of cold and heated (70°C) water, were used for measuring the biofilm formation potential (BFP) of these SSFs and the growth of *L. pneumophila* in the biofilms on glass and chlorinated polyvinylchloride (CPVC). A silicone rubber tubing segment with *L. pneumophila* and associated microbiota was inserted in the warm-water supply pipe for inoculation of the BBM. The BfC was quantified by ATP analysis, total cell count (TCC), and HPCs. Terminal restriction fragment length polymorphism (T-RFLP) analysis and 16S rRNA gene cloning and sequencing were used to obtain information about the identities and abundances of the predominating bacteria in the biofilms. The collected data were analyzed to elucidate relationships between the SSF compositions, biofilm concentrations, and *L. pneumophila* colony counts in the biofilms.

TABLE 1 Quality characteristics of the SSFs during the test period

SSF[a]	Temp range (°C)	Avg value ± SD (no. of measurements) for indicated characteristic			
		TOC (mg liter^{-1})	ATP (ng liter^{-1})	AOC (μg acetate-C eq liter^{-1})	AOC/TOC (μg acetate-C eq/mg C)
I	13.3–16.0	2.4 ± 0.3	2.0 ± 1.1 (9)	3.7 ± 0.9 (3)	1.6 ± 0.4 (3)
ID	13.5–19.0	2.4 ± 0.3	1.7 ± 0.8 (11)	3.7 ± 0.3 (3)	1.6 ± 0.1 (3)
II	10.7–15.2	2.3 ± 0.3	1.9 ± 0.8 (11)	3.7 ± 1.3 (2)	2.1 ± 1.1 (2)
IIIA	9.7–21.8	3.1 ± 0.3 (4)	4.6 ± 1.9 (10)	14.2 ± 2.7 (4)	4.6 ± 1.0 (4)
IIIB	5.6–19.5	3.3 ± 0.5 (32)	2.5 ± 1.2 (12)	22.0 ± 11.8 (2)	6.3 ± 1.9 (2)
IIID	8.8–19.1	3.2 ± 0.5 (11)	2.5 ± 0.8 (12)	10.8 ± 1.8 (2)	4.9 ± 0.6 (2)
IV	8.4–17.7	1.17 ± 0.15 (14)	1.4 ± 0.6 (12)	6.6 (1)[b]	7.1 (1)
IVD	7.9–18.3	1.16 ± 0.18 (14)	1.5 ± 0.8 (12)	4.3 ± 0.3 (1)	4.3 ± 0.3 (1)

[a]SSFs I, II, III, and IV are finished water of supplies I, II, III and IV. SSFs ID, IIID, and IVD are drinking water at a location in the respective distribution system. SSF III was tested in two time periods, designated SSF IIIA and SSF IIIB; SSF IIID is from the second time period.
[b]Similar to the average concentration over a 5-year period (9).

TOC and AOC concentrations in the slow sand filtrates. The SSFs, which had temperatures ranging from 2 to 20°C and were distributed without disinfectant, were characterized by low turbidity (average of ≤0.05 formazin turbidity units [FTU]), low concentrations of iron, manganese, and ammonia, low HPCs, absence of coliforms (Table S1), and low ATP concentrations (Table 1). The average natural organic matter (NOM) concentrations in the SSFs in the period of the investigation, measured as TOC, varied from 1.1 mg C liter^{-1} (SSF IV) to 3.2 mg C liter^{-1} (SSF III), and the average AOC concentrations ranged from 3.7 μg ac-C eq liter^{-1} (SSF I) to 22 μg ac-C eq liter^{-1} (SSF III) (Table 1). Storage of samples from SSFs I and II during 6 h at 70°C, simulating the conditions in the BBM boiler, caused a 3.3 (±0.4)-times AOC increase to an average AOC/TOC ratio of 6.3 (±0.6) μg ac-C eq mg^{-1} C. Heating of samples from SSFs III and IV caused a 2.6 (±0.2)-times AOC increase and revealed a water temperature dependence of the AOC/TOC ratio (Fig. 1). From the temperature-dependent mixing ratios of cold and heated water (i.e., mixing ratio to achieve a temperature of 37°C), it was derived that the AOC in the warm water was 2.0 times (SSFs I and II) and 1.7 times (SSFs III and IV) the AOC in the cold water. Compounds promoting the growth of test strain *Spirillum* sp. strain NOX accounted for ≥90% of the AOC concentrations in the SSFs before and after heating (Fig. 1).

Biofilm formation and relationship with TOC and AOC. The BfC values (pg ATP cm^{-2}) on glass and CPVC in the BBMs supplied with the SSFs increased with exposure time and reached maximum levels ranging from approximately 400 pg ATP cm^{-2} (on glass exposed to SSF IVD [at a location in the distribution system of SSF IV]) (Fig. 2H) to 5,000 pg ATP cm^{-2} (on CPVC exposed to SSF IIIA [first of two test series of SSF III]) (Fig. 2D). Most variation was observed within 100 days of exposure, and the average BfC after more than about 100 days was defined as the biofilm formation potential (BFP) of

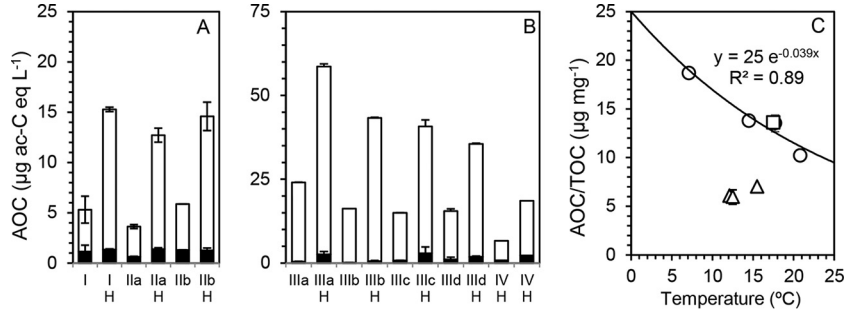

FIG 1 (A, B) AOC concentrations in SSFs I and II (A) and SSFs III and IV (B) before and after heating (H) for 6 h at 70°C. Black bars, strain P17; white bars, strain NOX. Error bars show standard deviations. (C) Relationship between the AOC/TOC ratios in heated water and the temperatures for SSFs I and II (△), SSF III (○), and SSF IV (□). The equation gives the relationship for SSFs III and IV (solid line).

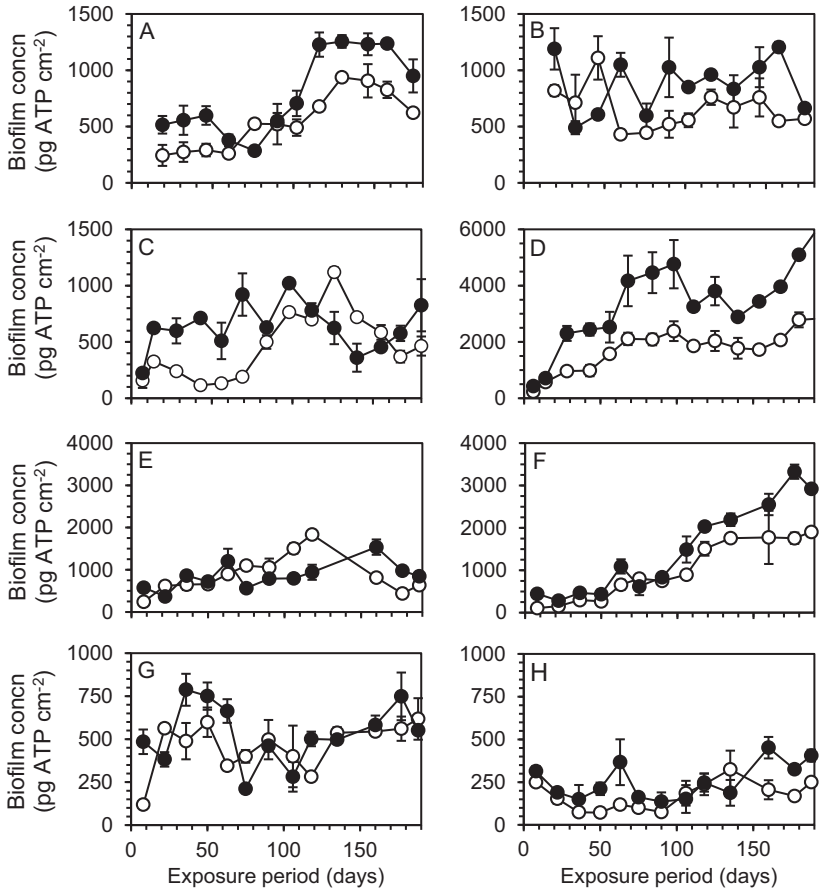

FIG 2 Biofilm concentrations on glass (○) and CPVC (●) in boiler biofilm monitors (BBMs) supplied with slow sand filtrates (SSFs) during approximately 6 months. (A) SSF I (finished water); (B) SSF ID (distribution system); (C) SSF II (finished water); (D) SSF IIIA (finished water, first test series); (E) SSF IIIB (finished water, second test series); (F) SSF IIID (distribution system); (G) SSF IV (finished water); (H) SSF IVD (distribution system). Panels D and G include previously published data (15), with permission. Error bars show standard deviations.

the tested water. SSF III was tested for a second time after 1 year (SSF IIIB), in combination with drinking water from a location in the distribution system (SSF IIID). The BfCs on glass and CPVC exposed to SSF IIIB (Fig. 2E) remained below the level observed with SSF IIIA, but from day 124 to day 137, the BBM was not operated (no flow) due to boiler failure. The average CPVC-to-glass ratio of the BFPs was 1.4 ± 0.3, with significantly higher BFPs on CPVC at five locations (Table 2). The BFP of SSF I at a location in the distribution system (SSF ID) did not differ significantly from the BFP of SSF I, but SSF IVD had a significantly lower BFP ($P < 0.005$) than SSF IV. The BFPs on glass and CPVC showed significant linear relationships with the TOC concentrations in the SSFs ($P < 0.05$; $R^2 \geq 0.67$) and the AOC concentrations in the warm water ($P < 0.002$; $R^2 \geq 0.88$) (Fig. 3; Table S2). The logarithmic means of the TCCs of the biofilm, which were analyzed less frequently than the ATP concentrations, ranged from 6.6 log cells cm^{-2} on glass exposed to SSFs I and II to 7.4 log cells cm^{-2} on glass and CPVC exposed to SSF III (Table 2). The logarithmic mean of the HPCs was ≤7 log CFU cm^{-2} and <10% of the TCCs. The HPCs, TCCs, and ATP concentrations in the biofilms showed significant correlations, but the relationships were weak (low R^2 values) (Table 3). The average concentrations of Fe and Mn in the biofilms in most cases were <10 mg m^{-2} (Fe) and <1 mg m^{-2} (Mn). The highest Fe concentrations (about 25 mg Fe m^{-2}) were observed on CPVC exposed to SSF IV and to SSF IIID where the water had passed cast iron pipes. At a few locations, the concentrations of Fe and/or Mn were significantly higher on CPVC than on glass.

TABLE 2 Characteristics of biofilms on glass and CPVC in the BBMs supplied with SSFs I, II, III, and IV

SSF, biofilm supporting material[a]	Avg value ± SD for indicated characteristic[b]							
	BFP (pg ATP cm^{-2})	LpC (log CFU cm^{-2})	TCC (log N cm^{-2})	ATP/TCC (fg $cell^{-1}$)	HPC (log CFU cm^{-2})	HPC/TCC (%)	Fe (mg m^{-2})	Mn (mg m^{-2})
I								
Glass	795 ± 140	3.35 ± 0.25	6.6 ± 0.1	0.09 ± 0.02	5.7 ± 0.1	13 ± 4	5.4 ± 3.8	0.27 ± 0.25
CPVC	1,180 ± 140[c]	1.3 ± 0.25[c]	7.0 ± 0.02	0.07 ± 0.05	6.1 ± 0.2	18 ± 8	4.3 ± 2.6	0.33 ± 0.17
ID								
Glass	645 ± 125	3.1 ± 0.55	7.0 ± 0.1	0.10 ± 0.05	6.0 ± 0.3	19 ± 9	1.8 ± 0.6	0.18 ± 0.16
CPVC	925 ± 220[c]	3.1 ± 0.25	7.0 ± 0.2	0.10 ± 0.05	6.3 ± 0.3	17 ± 11	3.9 ± 2.8	0.63 ± 0.35[c]
II								
Glass	660 ± 253	3.75 ± 0.5	6.6 ± 0.3	0.09 ± 0.01	5.6 ± 0.5	9 ± 6	4.5 ± 1.3	0.14 ± 0.11
CPVC	605 ± 180	4.9 ± 0.2[c]	6.9 ± 0.3	0.08 ± 0.04	5.8 ± 0.4	6 ± 4	6.3 ± 4.4	0.34 ± 0.44
IIIA								
Glass	2,090 ± 270	4.8 ± 0.4	7.4 ± 0.3	0.12 ± 0.07	6.6 ± 0.3	25 ± 18	3.7 ± 2.5	0.19 ± 0.12
CPVC	3,980 ± 795[c]	3.8 ± 0.5[c]	7.4 ± 0.4	0.23 ± 0.14	7.0 ± 0.1	55 ± 43	7.2 ± 4.5[c]	2.1 ± 2.9[c]
IIIB								
Glass	670 ± 160	2.8 ± 1.0	6.9 ± 0.4	0.10 ± 0.02	6.6 ± 0.04	29 ± 5	4.7 ± 1.4	0.11 ± 0.05
CPVC	1,150 ± 370	4.4 ± 0.3[c]	7.1 ± 0.2	0.07 ± 0.03	6.5 ± 0.5	13 ± 1	7.8 ± 1.7[c]	0.47 ± 0.43
IIID								
Glass	1,740 ± 260	3.4 ± 0.3	7.2 ± 0.02	0.07 ± 0.03	6.6 ± 0.4	17 ± 3	17.4 ± 8.7	0.84 ± 0.59
CPVC	2,605 ± 510[c]	2.0 ± 0.5[c]	7.4 ± 0.1	0.06 ± 0.02	6.7 ± 0.3	18 ± 7	15.7 ± 3.9	0.35 ± 0.28
IV								
Glass	490 ± 115	2.0 ± 0.7	7.0 ± 0.2	0.07 ± 0.03	5.8 ± 0.4	6 ± 2	9.3 ± 1.9	0.45 ± 0.07
CPVC	575 ± 105	1.9 ± 0.7	7.1 ± 0.24	0.04 ± 0.01	5.8 ± 0.4	6 ± 3	18.1 ± 6.6[c]	0.96 ± 0.55[c]
IVD								
Glass	230 ± 55	3.8 ± 0.5	6.7 ± 0.24	0.03 ± 0.02	5.6 ± 0.2	5 ± 2	5.9 ± 2.3	0.52 ± 0.31
CPVC	325 ± 110[c]	1.8 ± 0.6[c]	6.9 ± 0.23	0.03 ± 0.02	5.9 ± 0.2	11 ± 4	10.2 ± 5.2[c]	0.51 ± 0.41

[a]SSF ID, slow sand filtrate (finished water) of supply I tested at a distribution system location; SSF IIIA, supply III, first test series; SSF IIIB, second test series; SSF IIID, SSF IIIB at a distribution system location.
[b]BFP, biofilm formation potential, average biofilm concentration after >90 to 100 days of exposure; LpC, *Legionella* colony count, log average after >60 days of exposure; TCC, total cell count ($n = 4$ to 7); HPC, heterotrophic plate count ($n = 6$ to 12).
[c]Concentration on CPVC was significantly different ($P < 0.05$) from concentration on glass.

Growth of *L. pneumophila* in the biofilm. *L. pneumophila* multiplied in all biofilms on glass and CPVC, in most cases after about 20 to 30 days (Fig. 4). The maximum *Legionella pneumophila* colony count (LpC) in the biofilm varied from 2×10^3 CFU cm^{-2} (SSF IV) to 1.5×10^5 CFU cm^{-2} (SSF IIIA) on glass and from 6×10^2 CFU cm^{-2} (SSF I) to 1.4×10^5 CFU cm^{-2} (SSF II) on CPVC. The logarithmic mean of LpC was significantly higher on glass than on CPVC at four locations, three of which had a significantly lower BFP on glass (Table 2). Only with SSF II was the LpC significantly higher on CPVC. The LpC/BfC ratios, with median values for glass and CPVC ranging from 17 (SSF IIID) to 107

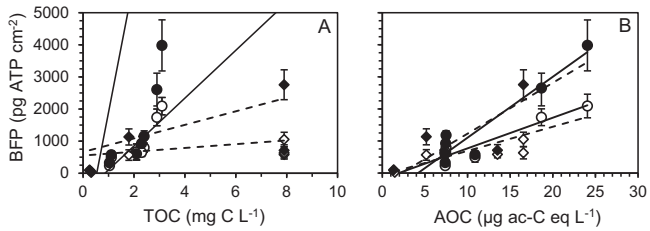

FIG 3 Relationship between the steady-state biofilm concentrations (biofilm formation potentials [BFPs]) on glass (open symbols) or CPVC (solid symbols) in the BBMs and the TOC concentrations (A) or the assimilable organic carbon (AOC) concentrations (B) in the warm water. Circles show the data and solid lines the relationships for the SSFs, and broken lines show the relationships with inclusion of the data for treated groundwater (diamonds) (Table S2) (9). Error bars show standard deviations.

TABLE 3 Correlations and linear regression-based relationships between log-transformed data of biofilm characteristics and LpCs in biofilms in BBMs supplied with the SSFs of four surface water supplies

Parameters (no. of data pairs)	*P* value	Slope ± SE	R^2
TCC vs HPC (55)	$<10^{-6}$	0.83 ± 0.14	0.40
TCC vs ATP (64)	$<10^{-10}$	0.67 ± 0.08	0.51
HPC vs ATP (92)	$<10^{-16}$	0.56 ± 0.05	0.56
LpC vs TCC (47)[a]	0.79	-0.11 ± 0.43	<0.01
LpC vs HPC (82)[a]	0.16	0.32 ± 0.22	0.02
LpC vs ATP (252)[a]	$<10^{-3}$	0.68 ± 0.19	0.05
LpC vs BFP (16)[b]	0.54	0.56 ± 0.88	0.03

[a]Data from the period before growth of *L. pneumophila* not included.
[b]Average concentrations of *L. pneumophila* (LpC) and ATP (BFP) in the steady-state biofilms.

CFU/pg ATP (SSF I), were significantly higher on glass than on CPVC in the biofilms in the BBMs supplied with SSFs I ($P < 10^{-6}$), IIIA ($P < 10^{-6}$), IIID ($P < 10^{-3}$), and IVD ($P < 10^{-4}$). The LpC/TCC ratios (all data) were also significantly higher on glass (median value, 0.025%) than on CPVC (median value, 0.004%) ($P < 0.05$). Apparently, growth of *L. pneumophila* was more efficient on glass than on CPVC, except for SSF II, where the LpC/BfC ratio was significantly higher on CPVC than on glass ($P < 0.01$). The LpCs correlated significantly with the BfC values (all data, period before growth of *L.*

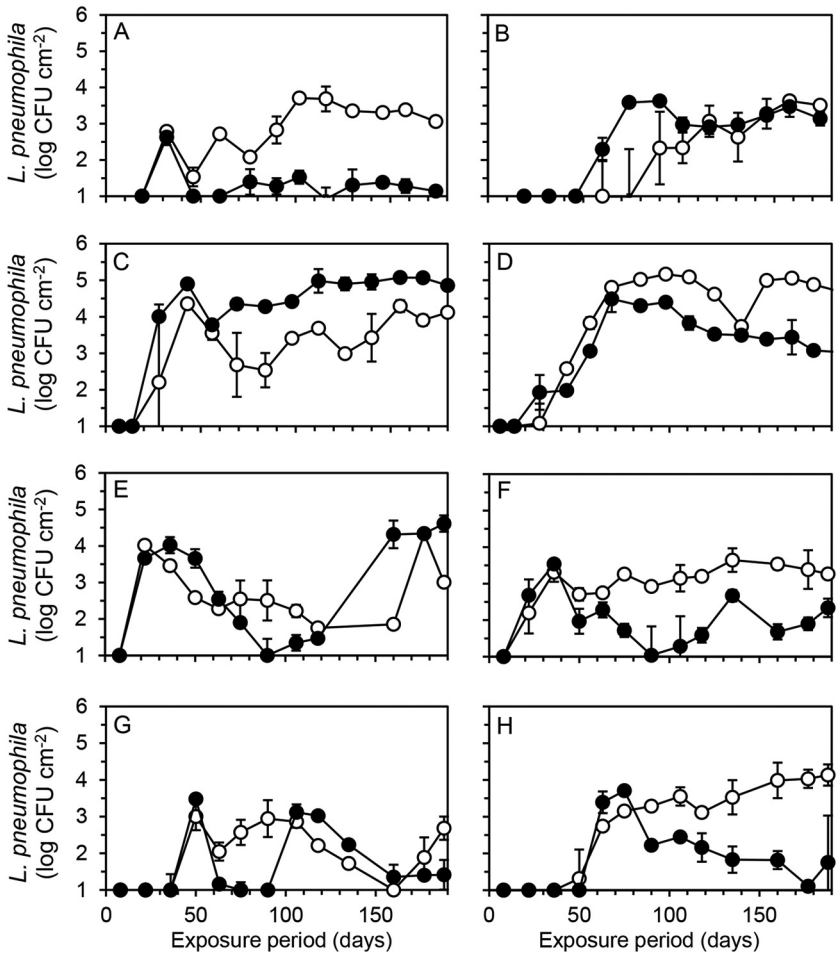

FIG 4 *L. pneumophila* colony counts (LpC) in the biofilms on glass (○) and CPVC (●) in the BBMs supplied with SSFs during approximately six months. (A) SSF I; (B) SSF ID; (C) SSF II; (D) SSF IIIA; (E) SSF IIIB; (F) SSF IIID; (G) SSF IV; (H) SSF IVD. Panels D and G include previously published data (15), with permission. LpC values below the detection limit are shown as log LpC = 1. Error bars show standard deviations.

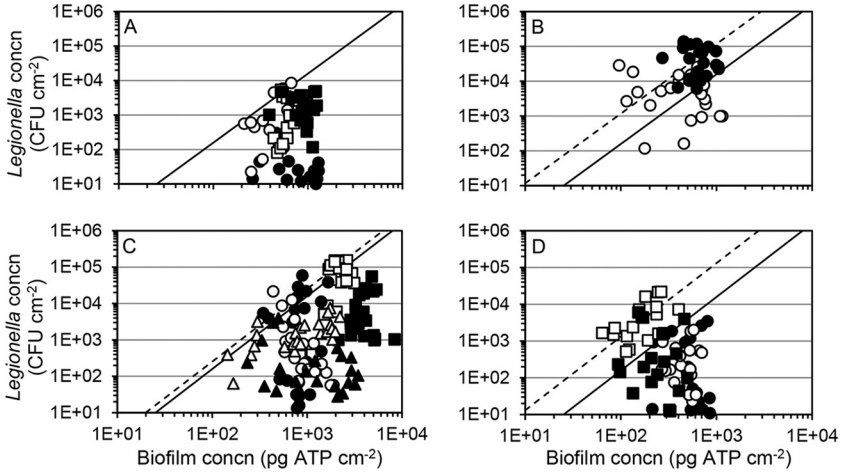

FIG 5 *L. pneumophila* colony counts (LpC) at the associated biofilm concentrations (BfCs) on glass (open symbols) and CPVC (solid symbols) in BBMs supplied with slow sand filtrates. (A) SSFs I (○,●) and ID (□, ■); (B) SSF II; (C) SSFs IIIA (□, ■), IIIB (○, ●), and IIID (△, ▲); (D) SSFs IV (○, ●) and IVD (□, ■). The solid lines represent the following relationship: log LpC = 2 log BfC − 1.8 (see Materials and Methods). The broken lines are based on average values for log *b*, calculated for SSF II on CPVC (−0.93), SSF IIIA on glass (−1.6), and SSF IVD on glass (−0.88) (see Fig. S4).

pneumophila excluded) (Table 3) but varied by several log units at the associated BfC values (Fig. 5), and the relationship was very weak ($R^2 = 0.05$). The logarithmic means of the LpCs did not correlate with the BFPs on glass and CPVC (Table 3). However, in the biofilms exposed to samples from SSFs I and III, the high LpC values with high CFU/pg ATP ratios fitted with the relationship (solid line) derived in a previous study (9). The LpC/BfC ratios of a number of colony counts in the biofilms exposed to samples from SSFs II and IVD exceeded the relationship presented by the solid line (Fig. 5).

Free-living amoebae in the biofilms. A total of 24 biofilm samples were examined for the presence of *Acanthamoeba* spp. and *Vermamoeba vermiformis* because these organisms are potential hosts for *L. pneumophila*. *Acanthamoeba* spp. were not detected in any of these samples (<0.5 cell equivalents [cell eq] cm^{-2}). *V. vermiformis* was detected in 19 samples at concentrations ranging from 0.7 to 384 cell eq cm^{-2} (Table S3). The *V. vermiformis* concentration (VvC) did not correlate with the BfC. The amoeba was not detected in the biofilms exposed to SSF I (CPVC) sampled on day 102, SSF ID sampled on day 46, or SSF IVD sampled on day 50. The LpCs in these samples were <50 CFU cm^{-2} or not detectable (<14 CFU cm^{-2}) (Fig. 4A, B, and H). On CPVC, the LpC correlated significantly with the VvC (Fig. 6), but the relationship was weak.

Predominating biofilm bacteria. T-RFLP analysis by capillary electrophoresis was applied to obtain an indication of the diversity of the predominating bacteria in the biofilms. The 93% to 98% similarity of the T-RFLP profiles observed in a number of

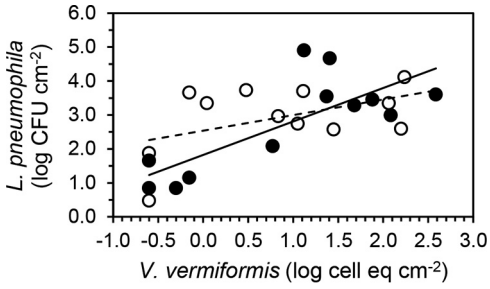

FIG 6 The *L. pneumophila* colony count (LpC) in relation to the *V. vermiformis* concentration (VvC) in the biofilm on glass (○) and CPVC (●). For glass, $R^2 = 0.23$ and $P = 0.12$ (broken line); for CPVC, $R^2 = 0.59$ and $P = 0.003$ (solid line); for all data, $R^2 = 0.41$ and $P = 7.4 \times 10^{-4}$.

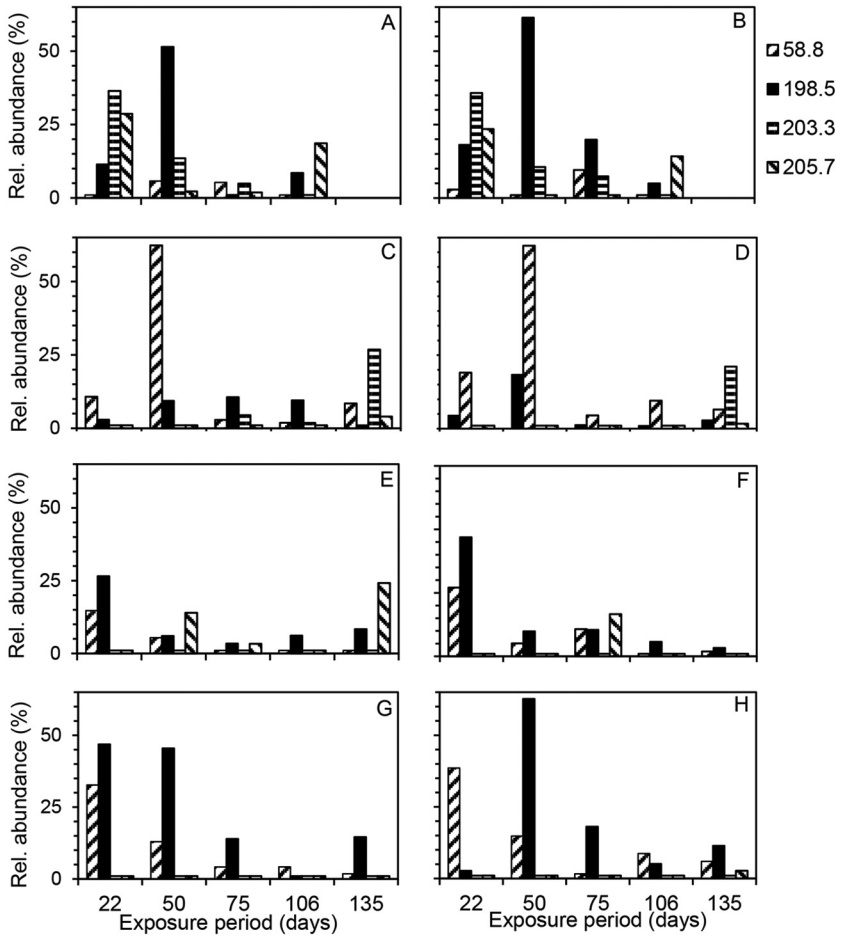

FIG 7 Relative abundances of the bacteria with T-RFs of 58.8 nt (*M. discipulorum*), 198.5 nt (*P. aquaticus*), 203.3 nt (*C. manganoxidans*), and 205.7 nt (uncultured *Rhodocyclaceae* bacterium) in biofilms on glass and CPVC in BBMs supplied with SSFs. (A) SSF IIIB, glass; (B) SSF IIIB, CPVC; (C) SSF IIID, glass; (D): SSF IIID, CPVC; (E) SSF IV, glass; (F) SSF IV, CPVC; (G) SSF IVD, glass; (H) SSF IVD, CPVC. SSF IIID and SSF IVD indicate locations in the respective distribution systems.

samples collected on the same day from the biofilms on glass and CPVC (Fig. S2) demonstrated the high reproducibility of this analysis and of the experimental conditions within the BBM. In 67 biofilm samples, a total of 85 different terminal restriction fragments (T-RFs), each accounting for ≥1.0% of the total peak surface of an individual sample, was observed. In individual samples, the numbers of T-RFs ranged from 3 to 22, with the lowest numbers after ≤50 days of exposure (Table S4). The number of T-RFs (≥1.0%) at each location ranged from 27 (SSFs I and II) to 40 (SSF IIIA). Most (75%) T-RFs had a total relative abundance (all samples) of <1%, and five T-RFs, each with a total relative abundance of >5%, accounted for approximately 35% of the total abundance. One or several of these T-RFs (with lengths of 58.8 nucleotides [nt], 198.5 nt, 203.3 nt, and 205.7 nt) constituted ≥50% of the bacterial abundance in the biofilms sampled after ≤50 days of exposure to SSFs II, IIIB, IIID, IV, and IVD (Fig. 7; Fig. S3). Apparently only a few types of bacteria, the primary colonizers, prevailed in these young biofilms. To identify these predominating bacteria, the sequences of a total of 424 clones retrieved from 14 biofilm samples from the BBMs supplied with SSFs IIIB, IIID, IV, and IVD were analyzed. Members of the *Alphaproteobacteria* and the *Betaproteobacteriales* (*Gammaproteobacteria*), previously classified as *Betaproteobacteria*, accounted for about 88% of the bacterial abundance in these samples (Table 4). The *Betaproteobacteriales* were predominated by *Piscinibacter*, *Caldimonas*, *Methyloversatilis*, and an uncultured *Rhodocyclaceae* bacterium. These bacteria accounted for >50% of the se-

TABLE 4 Identities and relative abundances of bacteria predominating in biofilms on glass and CPVC in BBMs supplied with SSFs of surface water supplies III and IV[a]

Classification[b]	No. (%) of clones in SSFs: IIIB and IIID (n = 6 samples)	IV and IVD (n = 8 samples)	Total (n = 14 samples)	No. (%) of: OTUs[c]	Positive samples
Proteobacteria, *Alphaproteobacteria*					
Reyranellales, *Reyranellaceae*					
Reyranella massiliensis	3 (2.1)	6 (2.2)	9 (2.1)	3 (1.9)	3 (21)
Rhizobiales, *Beijerinckiaceae*					
Bosea massiliensis[d]		4 (1.4)	4 (0.9)	2 (1.2)	2 (14)
Rhizobiales, *Hyphomicrobiaceae*					
Uncultured bacterium	1 (0.7)	3 (1.1)	4 (0.9)	4 (2.5)	2 (14)
Rhizobiales, *Xanthobacteraceae*					
Bradyrhizobium japonicum[d]	1 (0.7)	17 (6.1)	18 (4.2)	5 (3.1)	3 (21)
Pseudorhodoplanes sinuspersici	2 (1.4)	24 (8.6)	26 (6.1)	12 (7.4)	4 (29)
Uncultured bacterium[e]		5 (1.8)	5 (1.2)	1 (0.6)	1 (7)
Rhodoplanes sp. strain laus-2[f]	7 (4.8)	49 (17.6)	56 (13.2)	18 (11.1)	8 (57)
Uncultured bacterium[g]	20 (13.8)	8 (2.9)	28 (6.6)	13 (8.0)	5 (36)
Uncultured bacterium		3 (1.1)	3 (0.7)	3 (1.9)	2 (14)
Rhodobacterales, *Rhodobacteraceae*					
Uncultured bacterium		1 (0.4)	1 (0.2)	1 (0.6)	1(7)
Sphingomonadales, *Sphingomonadaceae*					
Novosphingomonas aromaticivorans	1 (0.7)		1 (0.2)	1 (0.6)	1 (7)
Proteobacteria, *Alphaproteobacteria*					
Uncultured bacterium	2 (1.4)	2 (0.7)	4 (0.9)	2 (1.2)	2 (14)
Proteobacteria, *Gammaproteobacteria*					
Betaproteobacteriales,[h] *Burkholderiaceae*					
Caldimonas manganoxidans[d]	33 (22.8)	1 (0.4)	34 (8.0)	9 (5.6)	5 (36)
Comamonas, uncultured bacterium	12 (8.3)	10 (3.6)	22 (5.2)	9 (5.6)	4 (29)
Limnobacter thiooxidans[d]		6 (2.2)	6 (1.4)	2 (1.2)	2 (14)
Piscinibacter aquaticus[d]	21 (14.5)	61 (21.9)	82 (19.3)	15 (9.3)	13 (93)
Schlegelella aquatica		6 (2.2)	6 (1.4)	5 (3.1)	1 (7)
Uncultured bacterium	3 (2.1)	3 (1.1)	6 (1.4)	2 (1.2)	3 (21)
Betaproteobacteriales, *Rhodocyclaceae*					
Methyloversatilis discipulorum[d]	3 (2.1)	20 (7.2)	23 (5.4)	8 (4.9)	7 (50)
Uncultured *Rhodocyclaceae* bacterium[i]	9 (6.2)	18 (6.5)	27 (6.4)	9 (5.5)	6 (43)
Betaproteobacteriales, TRA3-20	2 (1.4)	5 (1.8)	7 (1.7)	6 (3.7)	4 (29)
Salinisphaerales, *Solimonadaceae*					
Hydrocarboniphaga daqingensis		1 (0.4)	1 (0.2)	1 (0.6)	1 (7)
Acidobacteria, *Blastocatellia* (subgroup 4)	6 (4.1)	5 (1.8)	11 (2.6)	5 (3.1)	4 (29)
Actinobacteria, *Thermoleophilia*, *Gaiellales*	1 (0.7)		1 (0.2)	1 (0.6)	1 (7)
Cyanobacteria, *Melainabacteria*, *Obscuribacterales*	2 (1.4)	7 (2.5)	9 (2.1)	6 (3.7)	4 (29)
Gemmatimonadetes, *Gemmatimonadetes*					
Gemmatimonadales, *Gemmatimonadaceae*	6 (4.1)	6 (2.2)	12 (2.8)	3 (1.9)	5 (36)
Planctomycetes, *Phycisphaerae*	2 (1.4)	4 (1.4)	4 (0.9)	3 (1.9)	3 (21)
OM190		1 (0.4)	3 (0.7)	2 (1.2)	3 (21)
Unclassified bacteria	8 (5.5)	3 (1.1)	11 (2.6)	11 (6.8)	6 (43)
Total	145 (100)	279 (100)	424 (100)	162 (100)	14 (100)

[a]Includes SSF IIIB (water supply III, second test); SSF IIID (SSF IIIB at a location in the distribution system), SSF IV (water supply IV), and SSF IVD (SSF IV at a location in the distribution system).
[b]According to SILVA Incremental Aligner (SINA) (version 1.2.11), version 132 (December 2017); classification to the species level based on ≥97% sequence similarity with type strain in NCBI BLAST.
[c]Clones with ≥99% sequence similarity (OTU code numbers are shown in Table S6).
[d]Also isolated from R_2A plates (results not shown).
[e]Clones with 96% sequence similarity to *P. sinuspersici*.
[f]Clones with ≥97% sequence similarity to strain *Rhodoplanes* sp. strain laus-2 with GenBank accession number DQ123621 (16).
[g]Clones with ≥97% sequence similarity to an uncultured *Xanthobacteraceae* bacterium with GenBank accession no. JQ323117.
[h]Previously classified as *Betaproteobacteria*.
[i]Clones with ≥98% sequence similarity to an uncultured *Rhodocyclaceae* bacterium with GenBank accession no. JQ278814.

quences retrieved from the biofilms on glass and CPVC exposed to SSF IIIB sampled on days 22 and 50, SSF IV sampled on day 22, and SSF IVD sampled on day 50 (Table S5). The similarity between the relative abundances of *Piscinibacter*, *Caldimonas*, and an uncultured *Rhodocyclaceae* bacterium in the biofilms on glass and CPVC exposed for 22 days to SSF IIIB is consistent with the T-RFLP results of these samples (Fig. 7). These

TABLE 5 Relative abundances of the predominating primary colonizing bacteria characterized by T-RF length in biofilm samples from the test locations

Avg T-RF length ± SD (nt)[a]	Identity[b]	Avg relative abundance (%) in biofilms from[c]: SSFs I + II (n = 19 samples)	SSFs III + IV (n = 48 samples)	Glass (n = 23 samples)	CPVC (n = 24 samples)	All (n = 67 samples)
198.5 ± 0.2	*Piscinibacter*	2.4	15.3[d]	11.0	15.8[e]	11.6
203.3 ± 0.1	*Caldimonas*	14.8	4.4	20.7	12.5	7.6
58.8 ± 0.1	*Methyloversatilis*	4.6	7.7	7.3	8.4	6.8
205.7 ± 0.2	Uncultured *Rhodocyclaceae* bacterium	<0.1	3.3[d]	9.1	5.4	2.4

[a]Average of data from the four SSFs.
[b]Identity derived from the relative abundances of the identified clones and the T-RFs in 14 biofilm samples collected from the BBMs supplied with SSFs IIIB, IIID, IV, and IVD.
[c]Relative abundance is the percentage of the T-RF peak surface relative to the total peak surface for the sample. Samples (collected at the same time from the same BBM) for which the T-RF was not detected on glass and CPVC were not included in the comparison of the effect of glass and CPVC.
[d]Significantly different from the relative abundance in biofilms exposed to SSFs I and II.
[e]Significantly different from the results for glass.

results revealed that *Piscinibacter* was represented by the T-RF of 198.5 nt, *Caldimonas* by the T-RF of 203.3 nt, *Methyloversatilis* by the T-RF of 58.8 nt, and an uncultured *Rhodocyclaceae* bacterium by the T-RF of 205.7 nt. The identification was confirmed by the relative abundances and presence-absence data in the other samples analyzed by T-RFLP (Table S7). The identified T-RFs showed that these four members of the *Betaproteobacteriales* constituted approximately 31% of the bacterial abundance in the biofilms in the BBMs supplied with SSFs III and IV (Table 5). *Caldimonas manganoxidans* had a high relative abundance in the SSF III biofilms, whereas *Methyloversatilis discipulorum* was more frequently observed in the SSF IV biofilms (Table 4). The T-RF of 198.5 nt showed a significantly lower relative abundance in biofilms of SSFs I and II in which the T-RF of 205.7 nt was not observed (Table 5; see also Fig. S3A). The T-RF of 203.3 nt was not observed in SSFs I and IV but accounted for 43% of the abundance in the biofilm in the BBM supplied with SSF II.

The *Alphaproteobacteria* were predominated by representatives of the *Rhizobiales* and accounted for 37.5% of the bacterial abundance in the biofilms on glass and CPVC exposed to SSFs III and IV (Table 4). The most abundant *Alphaproteobacteria* were a *Xanthobacteraceae* bacterium with ≥97% sequence similarity to *Rhodoplanes* strain laus-2 (GenBank accession no. DQ123621) and a *Pseudorhodoplanes* bacterium with ≥97% sequence similarity to *Rhodoplanes* strain laus-1 (GenBank accession no. DQ123619), both isolated from a hospital water system (16). These bacteria and *Bradyrhizobium* predominated in SSF IV biofilms, whereas bacteria related to uncultured *Xanthobacteraceae* bacteria were observed more frequently in biofilms exposed to SSF III (Table 4). Members of other phyla (e.g., *Acidobacteria* and *Gemmatimonadetes*) represented approximately 10% of the identified sequences. The T-RFs of these phyla and the *Alphaproteobacteria* were not identified.

Relationship between growth of *L. pneumophila* and primary colonizers. The more-than-2-log increase of LpCs between days 35 and 50 in the biofilms on glass and CPVC exposed to SSF IV (Fig. 4G) coincided with strong declines in the relative abundances of *Piscinibacter* (198.5 nt) and *Methyloversatilis* (58.8 nt) (Fig. 7; Table S5), suggesting that these bacteria served as prey for the amoebal host(s). This observation is consistent with the high relative abundances of these bacteria in the biofilms exposed to SSF IVD on days 22 and 50 in association with low LpCs (Fig. 4H; Table S5) and no detectable *V. vermiformis* on day 50 (Table S3), followed by a strong decline thereafter and growth of *L. pneumophila*. The strong decline (>90%) of the uncultured *Rhodocyclaceae* bacterium (205.7 nt) between day 22 and day 50 in the biofilm exposed to SSF IIIB (Fig. 7A and B; Table S5) in association with the growth of *L. pneumophila* (Fig. 4D) suggests that this bacterium was also a preferred prey for the amoebal host(s). In the biofilms exposed to SSF II, the T-RF of 203.3 nt (presumably *Caldimonas*) accounted for more than 65% of the bacterial abundance on day 14 (Fig. S3A) and

probably served as prey for the host amoeba(e), resulting in relatively high LpC values (Fig. 4C).

Taken together, under the test conditions (intermittent flow at 37°C and 6 months of exposure) the *L. pneumophila* colony counts show high variability at the associated biofilm concentrations. This variation is most likely due to changes in the biofilm microbiome and is also affected by the nature of the biofilm supporting material.

DISCUSSION

Relationships between TOC, AOC, and BFP. NOM present in the SSFs of the four SWSs had been exposed to various physicochemical processes (coagulation-sedimentation, oxidation, and adsorption) and biofiltration processes during dune passage, rapid filtration, granular activated carbon (GAC) filtration, and slow sand filtration (Fig. S1). The refractory nature of the remaining NOM is characterized by the AOC/TOC ratio (specific MGP), with the lowest values in SSFs I and II (Table 1). The average AOC/TOC ratios in SSFs III and IV pretreated by ozonation were about 3 times higher and increased with decreasing finished water temperature (11). The AOC increase after heating for 6 hours at 70°C shows that NOM present in the SSFs included labile fractions. In SSFs III and IV, the AOC/TOC ratios after heating also depended on the finished water temperature (Fig. 1C) and were approximately five times higher at 12°C than in treated groundwater of this temperature (9).

The overall relationships between the HPCs, TCCs, and ATP concentrations in the biofilms on the SSF-exposed surfaces (Table 3) were weaker (low values of R^2) than those in the biofilms on the surfaces exposed to treated groundwater (9). The nature of the biofilms on the SSF-exposed surfaces apparently differed from that of the biofilms on the surfaces exposed to treated groundwater, despite similar hydraulic conditions, exposed materials, and water temperatures in the BBMs. These differences may be related to seasonal changes in water composition and treatment conditions that do not occur in treated groundwater of approximately 12°C. The temperature variations of more than 10°C may also impact the microbial community composition of the SSFs, but data are not available. Biofilm formation was also affected by a boiler failure in the BBM supplied with SSF IIIB. The low initial BC at location SSF IIID, 24 km from the treatment plant, was caused by water mainly originating from SSF IV during the first month of operation (Fig. 2F).

Combining the BFP data of the SSFs with those of treated groundwater, which showed a similar AOC concentration range (9), substantiated the relationship between the BFP and AOC but not that between BFP and TOC (Fig. 3; Table S2), due to the large difference between the specific MGPs in SSFs III and IV and those in treated groundwater. An AOC concentration of 10 μg of ac-C eq liter^{-1} corresponds with a BFP on glass of approximately 700 pg ATP cm^{-2}. This BFP is clearly below the maximum biofilm concentration on glass (3,000 to 23,000 pg ATP cm^{-2}) exposed to continuously flowing tap water (270 liter h^{-1}) supplemented with 10 μg C liter^{-1} of acetate or maltose (17, 18). The difference may be attributed to (i) the low and intermittent water flow (6 liter h^{-1}) in the BBM system, (ii) protozoan grazing on the biofilm (see below), and (iii) a less ready availability of the AOC compounds compared to the availabilities of acetate and maltose. The strong relationship observed between the AOC concentrations and the ATP-based cumulative biomass production potentials (BPPs) of the SSFs showed that the AOC test strains utilized the same (groups of) compounds as the indigenous bacteria (11). More than 90% of the AOC after heating promoted the growth of *Spirillum* sp. strain NOX (Fig. 1), which is specialized in the utilization of low-molecular-weight carboxylic acids, including formate (19). The effect of heating therefore resembles the effects of chemical oxidation and UV light on NOM, which result in the formation of formic, acetic, malonic, and oxalic acids (20–22). Differences in the nature of the AOC compounds present in the finished water and in heated water most likely affected the relationship between the BFPs and the AOC concentrations of the warm water. Therefore, information about the concentrations of these compounds is needed for verification and elucidation of this relationship. At most locations, the BFP

was significantly higher on CPVC than on glass (Table 2), but CPVC does not release growth-promoting compounds after 8 weeks of contact with water (23). These elevated BFPs therefore may be attributed to enhanced bacterial attachment onto the hydrophobic CPVC surface (24).

Primary colonizing bacteria. The high relative abundances of *Piscinibacter* (T-RF of 198.5 nt), *Caldimonas* (T-RF of 203.3 nt), *Methyloversatilis* (T-RF of 58.8 nt), and an uncultured *Rhodocyclaceae* bacterium (T-RF of 205.7 nt) in the biofilms within 50 days of exposure to SSFs IIIB, IIID, IV, and IVD (Fig. 7; Table S5) show that these predominating bacteria were primary colonizers. Each of these bacteria was represented by a number of operational taxonomic units (OTUs) (Table 4; Table S6), demonstrating their ubiquity and affinity for the experimental conditions. The T-RF of 203.3 nt also predominated in biofilms on surfaces exposed to SSF II but not to SSF I, and the T-RFs of 198.5 nt and 58.8 nt accounted only for minor fractions in these biofilms (Fig. S3A). The bacteria associated with the T-RFs in these biofilms were not identified, but given the limited number of genera in the young biofilms in BBMs supplied with the SSFs and treated groundwater (9), it is conceivable that the T-RFs mentioned represented the same bacteria as identified in SSFs III and IV. The low AOC concentrations in the warm water imply that the primary colonizers possess substrate saturation constants at the microgram-per-liter level, combined with high growth rates when certain organic compounds are present and effective attachment properties. High substrate affinities have been observed in a variety of bacteria isolated from drinking water (19, 25). The relative abundances of the T-RF of 198.5 nt were significantly higher in biofilms exposed to SSFs III and IV with ozonation in the pretreatment than in biofilms exposed to SSFs I and II treated without ozonation (Table 5; Fig. S3B). Hence, ozonation may increase the availability of substrates preferred by *Piscinibacter aquaticus*, a methylotrophic member of the *Burkholderiaceae*. This bacterium and also members of the genus *Methyloversatilis* (*Rhodocyclaceae*) cannot utilize methane (26) but grow on C_1 compounds, including formate (27). Their prevalence in the biofilms on surfaces exposed to SSFs III and IV may therefore be related to the utilization of formate and other low-molecular-weight carboxylic acids that are produced from NOM by ozonation (20) and, most likely, also by heating of the water (Fig. 1). *C. manganoxidans* (T-RF of 203.3 nt), a moderately thermophilic poly-β-hydroxybutyrate (PHB)-accumulating bacterium, can utilize amino acids and a number of carbohydrates and carboxylic acids (formate not tested) (28). This broad nutritional versatility may enable its predominance in biofilms on surfaces exposed to SSFs II and III. However, data about the nutritional versatility and growth kinetics at the microgram-per-liter level are needed to elucidate the rapid growth of the primary colonizers in biofilms on surfaces exposed to warm tap water.

The decline of the abundance of the primary colonizers within 50 days of exposure (Fig. 7; Table S5) is most likely caused by protozoan grazing, because competition for substrate or space is unlikely at the low surface coverage by the biofilm bacteria (6.6 to 7.4 log bacteria cm^{-2}) (Table 2). The primary colonizers may be multiplying exponentially in microcolonies (29, 30) and serve as prey for protozoa even when the individual species abundance is less than 50 pg ATP cm^{-2}, the threshold concentration for growth of the host amoebae (9). The 20- to 100-times-higher LpC/BfC ratios (CFU pg^{-1} ATP) in the biofilms on glass compared to the ratios on CPVC in four of the tests suggest a more rapid turnover of prey bacteria on the hydrophilic glass than on the hydrophobic CPVC. Most of the *Alphaproteobacteria*, which predominated in the biofilms after >50 days of exposure (Table S5), are closely related to slowly growing bacteria isolated from cocultures with amoebae, e.g., *Rhodoplanes* strain laus-2 (GenBank accession no. DQ123621), *Pseudorhodoplanes sinuspersici* (*Rhodoplanes* strain laus-1 [GenBank accession no. DQ123619]), *Bradyrhizobium japonicum* (16, 31), *Reyranella massiliensis* (32), and *Bosea massiliensis* (33). Their association with amoebae in the biofilms is unclear; probably certain organic compounds present in the water are utilized for growth.

Host amoebae. *Vermamoeba vermiformis*, previously named *Hartmannella vermiformis* (34), is ubiquitous in drinking water (31) and has been identified as an amoebal host for *L. pneumophila* in many studies, mostly under experimental conditions, (e.g., see references 35–39). In the biofilms exposed to warm treated groundwater, the colony counts of *L. pneumophila* were strongly related to the concentrations of *V. vermiformis* (9). In the present study, the relationship was significant but weak (Fig. 6). The lack of correlation between the concentrations of this amoeba and the biofilm concentrations underlines the complexity of the processes involved with growth of *V. vermiformis* and *L. pneumophila*. Moreover, a quantitative assessment of the role of *V. vermiformis* as an amoebal host for *L. pneumophila* is complicated because (i) the PCR procedure used does not differentiate between trophozoites and cysts (36) and (ii) amoebal hosts other than *Acanthamoeba* spp. and *V. vermiformis* or free-living protozoa not serving as hosts may be present in the biofilms. On agar at 20°C, *V. vermiformis* attained its maximum growth rate at a concentration of $\geq 2 \times 10^7$ to 3×10^7 cells cm^{-2} of *Escherichia coli* and *Klebsiella aerogenes*, with half-saturation constants of 1×10^6 to 3×10^6 cells cm^{-2} (40). For several amoebae, the ingestion rates of *E. coli* at 7×10^6 cells cm^{-2} ranged from 15 to 440 prey $cell^{-1}$ h^{-1} (41), and variable yields have been reported for *V. vermiformis* amoebae feeding on different types of nongrowing bacteria present at high concentrations (40, 42). Hence, changes in the concentration and nature of prey bacteria (PB) most likely affected the growth of *V. vermiformis* in the BBM biofilms, but data about yield and ingestion rate in relation to PB concentrations in aquatic biofilms are lacking.

The LpC in the biofilm depends on the concentration of the host amoebae (HA) that become infected by this bacterium. Only 4.4% of the *Acanthamoeba castellanii* cells were infected after 1 h of exposure to 10^7 *L. pneumophila* cells ml^{-1} at a multiplicity of infection (MOI) of 100, and the infection rate was directly proportional to the MOI (43). Exposure of *V. vermiformis* to an LpC of 2×10^7 cm^{-2} (MOI of 1,000) at 35°C for 1 h resulted in 7.5% of amoebae with intracellular *L. pneumophila* cells (44). The LpC/VvC ratio in the biofilms was $<$50 CFU $cell\ eq^{-1}$ in 50% of the samples, with values of $>$1,000 CFU $cell\ eq^{-1}$ in 5 samples (SSF I, SSF II, and SSF IVD) (Fig. 6; Table S3), but these ratios may not represent the MOI because the presence of other host amoebae (not *Acanthamoeba* spp.) cannot be excluded. Therefore, only small and variable fractions of HA were infected in the biofilms on the SSF-exposed surfaces at LpC values ranging from $<$1,000 to 2×10^5 CFU cm^{-2}.

Relationship between *L. pneumophila* colony count and biofilm concentration. The LpC/BfC ratio in the biofilms depends on the BfC and the conversion efficiency (CE) (see equation 3, Materials and Methods). CE variation at a specific BfC may be associated with changes in the proportion and/or nature of PB caused by protozoan grazing, growth of amoeba-resistant bacteria, and/or water quality changes affecting the biofilm composition. These effects depend on the type of exposed material. Furthermore, the release of *L. pneumophila* several days after infection of HA grazing on the biofilm (36) causes nonsynchronous changes of BfC and LpC. A constant LpC/BfC ratio therefore requires a steady-state situation in biofilm concentration and composition. A steady-state situation is represented by the growth of *L. pneumophila* on plasticized PVC at 38°C with a constant water flow and an average BfC of 27 ($\pm$5.7) $\times$ 10^3 pg ATP cm^{-2} (39). For a period of 100 days, a value of -1.99 ± 0.16 was derived for log *b* (= log LpC $-$ 2 log BfC) (Fig. S4A), corresponding to a CE of 0.01. However, no information was collected about the concentration and nature of PB in that study. In the present study, log LpC $-$ 2 log BfC was highest in most cases within an exposure period of 100 days (Fig. S4B and S5). Obviously, the growth of *L. pneumophila* in BBM biofilms was most efficient in the non-steady-state situation when the primary colonizers constituted the major fraction of the biofilm (Fig. 7; Fig. S3). The more-than-10-times-higher LpC/BfC values in the biofilms on surfaces exposed to SSFs II and IVD (Fig. 5B and D) after $>$100 days correspond with log *b* values of -0.93 (SSF II) and -0.88 (SSF IVD) (Fig. S4B) and show that a low biofilm concentration can effectively support growth of *L. pneumophila*.

A significant correlation was reported between LpCs and TCCs in biofilms on various materials exposed to drinking water in a test rig, with up to a 3-log variation of LpC at a specific TCC value (45). These data also reveal more-than-proportional LpC increases at increasing TCCs, but no equation was given. Fig. S5 shows the data for LpCs in relation to TCCs collected in the present study in comparison with the relationships calculated with equation 4 (see Materials and Methods) using the overall average values of the ATP content of the cells (0.10 fg ATP cell^{-1} on glass; 0.05 fg ATP cell^{-1} on CPVC) and a CE value of 0.016. A higher ATP content of the cells and/or a higher CE corresponds with a higher LpC in relation to TCC. However, the increase of LpC in relation to the biofilm concentration (ATP or TCC) will shift from quadratic to linear at high percentages of HA infected by LpC. A detailed modeling of the complex and dynamic processes affecting the relationship between LpC and BfC requires more data on the biofilm composition and studies with pure bacterial cultures and *V. vermiformis* under defined conditions.

Practical implications. Slow sand filtration with extended physicochemical and biological pretreatment, including dune filtration, rapid sand filtration, and granular activated carbon filtration, does not reduce the BFP of warm drinking water to below the threshold level for *L. pneumophila* proliferation. The BFP was increased by the effect of heating of the water on the AOC concentration. The maximum LpC values in the biofilms on glass and CPVC in the BBM system (6×10^2 to 1.5×10^5 CFU cm^{-2}) were below the low prediction for the concentration of *L. pneumophila* in biofilms associated with infection by inhalation of shower aerosols (46). At a steady-state biofilm-to-water ratio of 10 CFU cm^{-2}/CFU liter^{-1}, these concentrations correspond with 500 to 10^4 CFU liter^{-1} (39), but 10-times-higher colony counts may occur with developing (non-steady-state) biofilms. Stagnation and plumbing materials may impact the colony count of *L. pneumophila* as well. These estimates exceed the maximum levels of $<$100 and $<$1,000 CFU liter^{-1} included in regulations in a number of European countries (47) and explain the detection of *L. pneumophila* in premise plumbing supplied with drinking water treated by slow sand filtration (12–14). The low AOC/TOC ratio of NOM in the SSFs and the effect of heating imply that physicochemical NOM removal would be required to reduce biofilm formation in premise plumbing to prevent growth of *Legionella*. The SSFs that are distributed without a disinfectant comply with stringent criteria for the microbiological quality of drinking water (Table S1), and further extension of water treatment therefore is not feasible. Consequently, water temperature (T) management ($T < 25$°C and $T > 60$°C), including the use of thermostatic valves to obtain the desired temperature at the tap, controlled hydraulic conditions, and the use of appropriate construction materials in contact with water, are essential to prevent proliferation of *L. pneumophila* in premise plumbing systems with drinking water when distributed without residual disinfectant. Still, in general, reducing the concentrations of AOC and NOM would result in lower biofilm concentrations and more-than-proportional reductions of *L. pneumophila* concentrations.

MATERIALS AND METHODS

Selected water supplies. Four SWSs with slow sand filtration as the final treatment were selected for the study. SWS I and SWS II apply pretreatment to river Meuse water, followed by dune filtration (average retention time of 60 days) and posttreatment, including lime softening, rapid sand filtration, and slow sand filtration. SWS III uses seepage water collected in a lake (depth, 6 to 14 m; surface area, 1.21 km^2) and treated with physicochemical and biological processes, including coagulation, rapid sand filtration, ozonation, pellet softening, granular activated carbon (GAC) filtration, and slow sand filtration. Water treatment at SWS IV includes pretreatment of river Rhine water and dune filtration (average retention time of 90 days), followed by posttreatments that include ozonation, softening, GAC filtration, and slow sand filtration. Treatment schemes are shown in Fig. S1 in the supplemental material, and Table S1 presents selected quality characteristics of the finished water (slow sand filtrates [SSFs]). The SSFs of the supplies were investigated, as well as drinking water at locations in the distribution systems of SWSs I, III, and IV (designated SSFs ID, IIID, and IVD). Details about these locations are presented in Table S8 in the supplemental material.

BBM system. The boiler biofilm monitor (BBM) system represents a worst-case situation for biofilm formation and growth of *L. pneumophila* in a warm tap water installation and has been described in detail in a previous publication (9). In brief, heated water (70°C) from an electric boiler (30 liter) was mixed with

the cold water to achieve a temperature of 38 ± 1°C. The warm water was supplied to two vertical glass columns (internal diameter, 2.5 cm; length, 60 cm) placed in a box with air temperature regulation to maintain a warm water temperature (37 ± 2°C) and prevent light access. Every 20 min, 1.5 to 2 liters of warm water was supplied to each of the columns over about 20 s. One column contained cylinders of glass (diameter, 1.8 cm; length, 1.6 cm; total surface, 17.4 cm^2) and the other CPVC pipe segments (diameter, 1.6 cm; length, 1.6 cm; total surface, 15 ± 1 cm^2). The BBM system was inoculated with *L. pneumophila* serogroup 1, sequence type 1, originating from a warm tap water installation in The Netherlands and cultured on pieces of silicone tubing in tap water at 37°C. A piece of silicone tubing with 10^4 to 10^5 CFU of the organism and associated microbiota, including *Vermamoeba vermiformis* (36), was inserted in the pipe directly after the thermostatic mixing valve, 1 to 2 weeks after the operational start of the BBM, and removed when *L. pneumophila* was observed in the biofilm on the exposed surfaces. Periodically, usually each 14 days, two cylinders were collected from each column and placed in 10 ml of autoclaved tap water contained in a capped glass tube. Within 24 h of storage at 5 ± 3°C, these samples were treated by low-energy ultrasound in a water bath (Branson sonication unit 5050). After each 2-min treatment, the water was taken from the tube and replaced with 10 ml of autoclaved tap water. Three ultrasonic treatments were applied to glass and six to CPVC. The suspensions obtained (30 ml for glass and 60 ml for CPVC) were used for microbiological and chemical analyses.

Microbiological analyses. Buffered charcoal yeast extract (BCYE) agar with antibiotics (48) was used to measure the colony counts of *Legionella*. Aliquots (0.1 ml) of the biofilm suspension or an appropriate decimal dilution in autoclaved tap water were spread over the surface of triplicate plates that were incubated at 36 ± 1°C for 7 days. The HPC was determined by using R_2A agar (49). Volumes of 0.05 ml of the collected biofilm suspension or an appropriate decimal dilution were spread over the surface of triplicate plates, followed by incubation at 25°C during 10 days. The TCC in the biofilm suspension was measured by using acridine orange staining and epifluorescence microscopy (50). The ATP concentrations of the suspension and the feed water were measured by using a bioluminescence assay as described elsewhere (51). The concentrations of *V. vermiformis* and *Acanthamoeba* spp. in the biofilm suspension were measured by quantitative PCR (qPCR) targeting the 18S rRNA gene (7). DNA was isolated from 1 to 10 ml of the biofilm suspension as previously described (18). For T-RFLP analysis, the 16S rRNA gene was PCR amplified using primer pair 8F-FAM (5′-AGAGTTTGATC[A/C]content-type=″gene″>TGGCTCAG-3′) and 1392R (5′-ACGGGCGGTGTGTACA-3′). T-RFLP profiles were generated with the ABI Prism genetic analyzer, model 310, with the GeneScan Analysis software as previously described (18). Length data for individual T-RFs were used for calculating their relative abundances in the samples investigated. To identify the predominating bacteria in biofilms, 16S rRNA gene cloning and sequencing were applied (18). The SILVA Incremental Aligner (SINA v1.2.11) with the SILVA database version 132 released on 13 December 2017 (https://www.arb-silva.de) were used for taxonomic classification of the sequences retrieved. In this database, the *Betaproteobacteria* have been reclassified as *Betaproteobacteriales*, an order of the *Gammaproteobacteria*. Sequences identified to the genus level were compared with the NCBI GenBank database by use of BLAST for species identification.

Chemical analyses. The concentrations of Fe and Mn in the biofilm suspension were measured by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Scientific Xseries 2 ICP-MS system (Thermo Fisher Scientific, Inc., USA) after destruction with nitric acid (pH < 2). The total organic carbon (TOC) concentration was measured by using a Shimadzu TOC-V analyzer (Shimadzu Corporation).

AOC. The concentrations of easily assimilable organic carbon (AOC) in the slow sand filtrates were measured by the simultaneous growth of *P. fluorescens* strain P17 and *Spirillum* sp. strain NOX in water samples heated to 60°C for 30 min in duplicate Erlenmeyer flasks and expressed in micrograms of acetate-C equivalents per liter (52). The introduction of a few micrograms of phosphate-P per liter with the inocula of the strains into the test samples ensured the utilization of more than 100 μg of C per liter without P limitation. The effect of heating on the AOC concentration was measured in samples after 6 h of storage at 70°C in the glass-stoppered Erlenmeyer flasks used for sampling and testing.

Conceptual model for growth of *Legionella* in aquatic biofilms. The growth of *L. pneumophila* in a biofilm on a water-exposed surface is the result of a succession of interacting dynamic processes, as follows: (i) attachment of indigenous bacteria to the exposed surface, (ii) biofilm formation by substrate uptake and growth of attached bacteria, (iii) consumption of prey bacteria (PB) by grazing amoebae, (iv) infection of host amoebae (HA) by *Legionella* bacteria, and (v) multiplication of *Legionella* within HA and lysis of HA, leading to (vi) release of *Legionella* bacteria in biofilm and water. In a previous investigation, a relationship was derived between the *Legionella* concentration (LpC, CFU cm^{-2}) and the biofilm concentration (BfC, pg ATP cm^{-2}) in BBMs supplied with treated groundwater at 37°C (9), as follows:

$$\log \mathrm{LpC} = a \log \mathrm{BfC} + \log b \quad (1)$$

with $a = 1.99 \pm 0.26$ and $\log b = -1.8 \pm 0.75$ ($R^2 = 0.88$) for BfC > 50 pg ATP cm^{-2} (threshold concentration for growth of LpC). Equation 1 can be written as follows:

$$\mathrm{LpC} = (\mathrm{BfC})^a \times \mathrm{CE} \quad (2)$$

where CE (10^b) represents the conversion efficiency of biofilm biomass (pg ATP cm^{-2}) to LpC (CFU cm^{-2}) by growth in HA. The HA concentration depends on the BfC fraction serving as PB. The value derived for *a* indicates a quadratic relationship between LpC and BfC that is consistent with the dependency of LpC on the HA concentration and the rate of infection of the HA that increases linearly with an increasing multiplicity of infection (see Discussion) (43, 44). From equation 2 with an *a* value of 2, it can be derived that

$$\mathrm{LpC/BfC} = \mathrm{BfC} \times \mathrm{CE} \quad (3)$$

which shows that the LpC/BfC ratio (CFU/pg ATP) depends on BfC and CE. Replacement of ATP by TCC in equation 2 gives

$$\mathrm{LpC} = (\mathrm{TCC}/N)^a \times \mathrm{CE} \quad (4)$$

where N is the number of cells corresponding with 1 pg of ATP.

Statistics. To determine the significance of differences between data, Student's *t* test was used for paired and unpaired samples with normally distributed data, eventually obtained after log transformation and verified with the Shapiro-Wilk test. For not-normally distributed data, the Wilcoxon signed-rank test was used for paired samples and the Mann-Whitney *U* test for independent samples. All testing was two tailed, with 95% confidence. The analyses were done with Real Statistics using Microsoft Excel 2010. Relationships between parameters were identified by linear regression analysis.

Accession number(s). Partial 16S rRNA gene sequences of the predominating uncultured bacteria have been deposited in NCBI GenBank under accession numbers MH930463 to MH930507.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01732-18.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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