

**Supplemental Material**

**of manuscript AEM02737-16:**

**Biofilm composition and threshold concentration for growth of *Legionella pneumophila* on surfaces exposed to flowing warm tap water without disinfectant**

Dick van der Kooij<sup>1#</sup>, Geo L. Bakker<sup>2</sup>, Ronald Italiaander<sup>1</sup>, Harm R. Veenendaal<sup>1</sup> and Bart A. Wullings<sup>1</sup>

<sup>1</sup>KWR Watercycle Research Institute

P.O. Box 1072, 3430 BB Nieuwegein, the Netherlands

<sup>2</sup>Vitens NV, P.O. Box 1205, 8001 BE, Zwolle, the Netherlands

# corresponding author

Dick van der Kooij

Email: [Dick.van.der.Kooij@kwrwater.nl](mailto:Dick.van.der.Kooij@kwrwater.nl)

Phone: #31 650 654 996

Table S1. Treated water quality at groundwater supplies A, B and C\*

<i>Characteristic</i>	<i>Supply A</i>	<i>Supply B</i>	<i>Supply C</i>
Temperature	10.0	12	13
Turbidity (FTE)	<0.2	<0.20	0.25
pH	7.96	7.7	7.9
Total hardness (mmol liter <sup>-1</sup> )	1.0	1.22	1.97
Conductivity (mS m <sup>-1</sup> )	22.2	48.4	38.7
Chloride (mg liter <sup>-1</sup> Cl)	14	29	9.9
Ammonia (mg liter <sup>-1</sup> NH <sub>4</sub> )	<0.05	<0.05	<0.04
Nitrate (mg liter <sup>-1</sup> NO <sub>3</sub> )	4.2	12	0.63
Iron (mg liter <sup>-1</sup> )	<0.02	0.02	0.07
Manganese (mg liter <sup>-1</sup> )	<0.01	<0.01	0.02
TOC (mg liter <sup>-1</sup> C)	<0.5	7.8	1.8
Heterotrophic plate count (CFU mL <sup>-1</sup> )**	2	23	<10
Coliforms 37°C (CFU 100 mL <sup>-1</sup> )	<1	<1	<1

\* mean values based on routine monitoring in a one-year period; \*\* Glucose yeast-extract agar, 3 days incubation at 22°C.

Table S2. Concentrations of *V. vermiformis*, *Acanthamoeba spp.* and *L. pneumophila* in the biofilm on glass and CPVC at four test locations

Water supply*	Day number	Material	<i>Biofilm</i> (pg ATP cm <sup>-2</sup> )	<i>V. vermiformis</i> (cell eq cm <sup>-2</sup> )**	<i>Acanthamoeba</i> spp. (cell eq cm <sup>-2</sup> )	<i>Legionella</i> (CFU cm <sup>-2</sup> )
A <sub>T</sub>	64	Glass	6	<0.5	<0.5	6
	64	CPVC	28	4	<0.5	90
	174	Glass	21	<0.5	<0.5	<6
	174	CPVC	58	<0.5	<0.5	<12
A <sub>D</sub>	34	Glass	54	<0.5	<0.5	6
	34	CPVC	70	0.5	<0.5	12
	118	Glass	65	<0.5	<0.5	6
	118	CPVC	102	<0.5	<0.5	26
B <sub>T</sub>	44	Glass	723	30	<0.5	<6
	44	CPVC	1772	11	<0.5	<12
	101	Glass	977	8	<0.5	253
	101	CPVC	2517	4	<0.5	41
C <sub>D</sub>	32	Glass	343	7	<0.5	7.1x10 <sup>4</sup>
	32	CPVC	289	174	<0.5	2.2 x10 <sup>4</sup>
	111	Glass	628	145	<0.5	4.6 x10 <sup>4</sup>
	111	CPVC	1128	96	<0.5	7 x 10 <sup>3</sup>

\* A<sub>T</sub>, treated water of water supply A; A<sub>D</sub>, location in distribution system of water supply A; B<sub>D</sub>, location in distribution system of water supply B; \*\* cell eq, cell equivalents.

Table S3. Identity of singleton bacterial clones from the biofilm on glass and CPVC at different periods (days, d) of exposure to drinking water type A (location in the distribution system, A<sub>D</sub>); type B (treated water, B<sub>T2</sub>) and type C (location in the distribution system, C<sub>D</sub>)

Classification*	A <sub>D</sub>		B <sub>T2</sub>		C <sub>D</sub>	
	d 118		d 41	d 138	d 60	
	glass	CPVC	glass	glass	glass	CPVC
<b>Proteobacteria, Alphaproteobacteria</b>						
<i>Rhizobiales, Bradyrhizobiaceae, Bradyrhizobium</i> (> 99)						1
<i>Rhizobiales, Hyphomicrobiaceae, Hyphomicrobium</i> (97)		1				
<i>Rhizobiales, Xanthobacteraceae, Xanthobacter</i> (> 99)	1					
<i>Rhizobiales, Uncultured bacterium</i> (98)				1		
<i>Rhodobacterales, Rhodobacteraceae, uncultured bacterium</i> (94)					1	
<i>Sphingomonadales, Sphingomonadaceae, Sphingomonas</i> (96)	1					
<b>Proteobacteria, Betaproteobacteria</b>						
<i>Burkholderiales, Comamonadaceae, uncultured bacterium</i> (94)					1	
<i>Burkholderiales, Comamonadaceae, uncultured bacterium</i> (90)				1		
<i>Burkholderiales, Comamonadaceae, Delftia</i> (> 99)	1					
<i>Nitrosomonadales, Nitrosomonadaceae, uncultured bacterium</i> (96)				1		
<i>Rhodocyclales, Rhodocyclaceae, uncultured bacterium</i> (92)			1			
Uncultured bacterium (93)				1		
Uncultured bacterium (80)					1	
<b>Proteobacteria Gammaproteobacteria</b>						
<i>Legionellales, Legionellaceae, Legionella, L. pneumophila</i> (> 99)					1	
<i>Xanthomonadales, Xanthomonadaceae, Lysobacter</i> (96)		1				
<b>Bacteroidetes, Cytophagia, Cytophagales, Cytophagaceae, Cytophaga</b> (89)						
<i>Acidobacteria, subgroup 4, uncultured bacterium</i> (89)					1	

\*classification with identity percentage at class, family or genus level based on SINA Alignment and at species level based on Blast.

Table S4. Identity and relative abundance of the predominating cultured biofilm bacteria at water types A<sub>D</sub>, B<sub>TD</sub> and C<sub>D</sub> on glass and CPVC\*

Classification**	Number (%) of isolates <sup>#</sup>	A <sub>D</sub>		B <sub>TD</sub> <sup>##</sup>		C <sub>D</sub>		Total abundance (%) <sup>§</sup>
		glass	CPVC	glass	CPVC	Glass	CPVC	
<b><i>Alphaproteobacteria</i></b>								
<i>Bradyrhizobium japonicum</i>	17 (29)	2 (2) <sup>‡</sup>		4 (4)			2 (1)	8 (30.8)
<i>Shingomonas</i> sp.	1 (1.7)						1	1 (3.8)
<b><i>Betaproteobacteria</i></b>								
<i>Piscinibacter aquaticus</i>	16 (27)	5 (4)	4 (4)	2(2)	4 (4)			15 (57.6)
<i>Methyloversatilis discipulorum</i>	10 (17)	2 (1)	1 (1)			2 (2)	1(1)	6 (23)
<i>Limnobacter thiooxidans</i>	6 (10)	2	3 (3)					5 (19.2)
<i>Curvibacter</i> sp.	8 (13.5)					1 (1)	2 (1)	3 (11.5)
<i>Caldimonas manganoxidans</i>	1 (1.7)			1				1 (3.8)
Number of isolates or samples	59 <sup>§</sup> (100)	6	6	6	4	2	2	26 <sup>§§</sup> (100)

\*A<sub>D</sub>, water supply A, location in distribution system; B<sub>TD</sub>, water supply B, treated water (T) and location in distribution system (D); C<sub>D</sub>, water supply C, location (laboratory) in distribution system; \*\*at species level  $\geq 97\%$  similarity with type strain; <sup>#</sup> in most samples only the dominating colony type was isolated; <sup>##</sup> combined results of locations T and D; <sup>§</sup> number and percentage of samples with organism; <sup>‡</sup> number of samples with organism; between brackets: number of samples with organism > 50% of colony count; <sup>§</sup> total number of isolates; <sup>§§</sup> total number of samples.

Table S5. Classification of clone sequences retrieved from biofilms on glass and CPVC in the boiler biofilm monitor exposed to warm tap water at groundwater supplies A (distribution system location A<sub>D</sub>), B (treated water B<sub>T2</sub>) and C (distribution system location C<sub>D</sub>)

Organism(s) with highest similarity (GenBank Accession number) classified according to lineage	Similarity (%)	A <sub>D</sub>		B <sub>T2</sub>		C <sub>D</sub>	
		day 118		day 41	day 138	day 60	
		glass	CPVC	glass	glass	glass	CPVC
<b><i>Proteobacteria: Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae</i></b>							
<i>Bradyrhizobium</i> sp. BTAi1 strain BTAi1 16S ribosomal RNA, complete sequence (NR_0743150/ <i>Bradyrhizobium denitrificans</i> strain IFAM 1005 16S ribosomal RNA gene, partial sequence ( NR_041827)	100/100						1
<b><i>Proteobacteria: Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae</i></b>							
Uncultured bacterium clone AS253O1hO5 16S ribosomal RNA gene, partial sequence (HM445062)/ Uncultured <i>Hyphomicrobiaceae</i> bacterium clone BBL-OTU1 16S ribosomalRNA gene, partial sequence (JQ791575);	100/99				4		
<i>Hyphomicrobium aestuarii</i> strain ATCC 27483 16S ribosomal RNA gene, partial sequence (NR_104954)/ <i>Hyphomicrobium vulgare</i> strain JCM 6889 16S ribosomal RNA gene,partial sequence (NR_104697)	99/99	2					
Uncultured bacterium clone 661192 16S ribosomal RNA gene, partial sequence (DQ404915) / Uncultured bacterium clone GW-2N1Ja_A05 16S ribosomal RNA gene, partial sequence (JX286014)	100/100		1				
<b><i>Proteobacteria: Alphaproteobacteria</i></b>							
Uncultured alphaproteobacterium clone BBL-OTU11 16S ribosomal RNA gene, partial sequence (JQ791585)/ Uncultured alphaproteobacteria clone 16S rRNA gene from clone QEDN10BH04 (CU927770)	99/99				1		
<b><i>Proteobacteria: Alphaproteobacteria, Rhizobiales, Pseudorhodoplanes</i></b>							
<i>Pseudorhodoplanes sinuspersici</i> strain RIPI 110 16S ribosomal RNA gene, partial sequence (JX500274.3)/ Alphaproteobacterium HTCC309 16S ribosomal RNA gene, partial sequence (AY429686) / Alphaproteobacterium CRIB-02 16S ribosomal RNA gene, partial sequence (DQ123619)	99/100/98			5	6		
<b><i>Proteobacteria: Alphaproteobacteria, Rhizobiales, Xanthobacteraceae</i></b>							
Alphaproteobacterium CRIB-04 16S ribosomal RNA gene, partial sequence (DQ123621)/ Uncultured bacterium clone HDB_SION961 16S ribosomal RNA gene,partial sequence (HM186203)	100/100	3	2		2	4	1

Table S5 Continued

Organism(s) with highest similarity (GenBank Accession number) classified according to lineage	Similarity (%)	A <sub>D</sub>		B <sub>T</sub> 2		C <sub>D</sub>	
		d 118		d 41	d 138	d 60	
		glass	CPVC	glass	glass	glass	CPVC
<b><i>Proteobacteria: Alphaproteobacteria, Rhizobiales, Xanthobacteraceae</i></b>							
Alphaproteobacterium CRIB-04 16S al RNA gene, partial sequence (DQ123621) / Uncultured bacterium clone HDB_SION961 16S ribosomal RNA gene, partial sequence (HM186203)	99/99	1	1				
Uncultured bacterium clone RO53 16S ribosomal RNA gene, partial sequence (EF219021)/ Alphaproteobacterium Shinshu-th1 gene for 16S rRNA (AB121772)	99/99				1		
<i>Xanthobacter flavus</i> strain ICS1 16S ribosomal RNA gene, partial sequence (JQ995474)/ <i>Xanthobacter</i> sp. Y8 16S ribosomal RNA gene, partial sequence (AY429710)	99/99	1					
<b><i>Proteobacteria: Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae</i></b>							
Uncultured Alphaproteobacterium gene for 16S ribosomal RNA, partial cds (AB604833)	99					1	
<b><i>Proteobacteria: Alphaproteobacteria, Rhodospirillales</i></b>							
<b><i>Rhodospirillales Incertae Sedis</i></b>							
<i>Reyranella massiliensis</i> strain 3B26 16S ribosomal RNA gene, partial sequence (HM048834)/ Uncultured bacterium clone NC5F1h3_14547 16S ribosomal RNA gene, partial sequence (JQ385362)	99/99	4	3	1			
Uncultured bacterium clone JE404 16S ribosomal RNA gene, partial sequence (KM841354)/ Uncultured <i>Acetobacteraceae</i> bacterium partial 16S rRNA gene, clone AMAD12 (AM935809)	100/99				1		
Uncultured bacterium clone JE404 16S ribosomal RNA gene, partial sequence (KM841354)/ Uncultured <i>Rhodospirillales</i> bacterium clone BBL 16S ribosomal RNA gene, partial sequence (JQ791590)	99/97				3		

Table S5 Continued

Organism(s) with highest similarity (GenBank Accession number) classified according to lineage	Similarity (%)	A <sub>D</sub>		B <sub>T</sub> 2		C <sub>D</sub>	
		d 118		d 41	d 138	d 60	
		glass	CPVC	glass	glass	glass	CPVC
<b><i>Proteobacteria: Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae</i></b>							
<i>Sphingopyxis indica</i> strain DS15 16S ribosomal RNA gene, partial sequence (NR_108185)/ <i>Sphingopyxis</i> sp. 113P3, complete genome (CP009452)	99/99	1	14				
Uncultured bacterium clone cpw1c216 16S ribosomal RNA gene, partial sequence (HM921106)	99	1					
<b><i>Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae</i></b>							
<i>Piscinibacter aquaticus</i> type strain (DQ664244)/ <i>Piscinibacter aquaticus</i> strain ERC1 16S ribosomal RNA gene, partial sequence (KP744146)	99/99	16	4	19	4		
<i>Piscinibacter aquaticus</i> strain ERC1 16S ribosomal RNA gene, partial sequence (KP744146)/ <i>Piscinibacter aquaticus</i> strain NBRC 102349 16S ribosomal RNA gene, partial sequence (NR_114061)	97/97					2	
Uncultured bacterium clone StLS43 16S ribosomal RNA gene, partial sequence (EU219006)/ <i>Methylibium petroleiphilum</i> PM1, complete genome (CP000555)	95/95					1	
Uncultured bacterium clone StLW2 16S ribosomal RNA gene, partial sequence (EU219038)/ <i>Piscinibacter aquaticus</i> strain NBRC 102349 16S ribosomal RNA gene, partial sequence ( NR_114061)	93/92				1		
<i>Ideonella dechloratans</i> partial 16S rRNA gene, isolate TOWS-108 (LN650473)/ <i>Ideonella</i> sp. IMCC1722 16S ribosomal RNA gene, partial sequence (DQ664241)	99/98					1	3
Uncultured bacterium clone BF-17 16S ribosomal RNA gene, partial sequence. (HQ609626)	95				1		
<i>Curvibacter fontana</i> type strain (AB120963)/uncultured <i>Curvibacter</i> sp. clone ESS-E8n 16S ribosomal RNA gene, partial sequence (FJ946584)	97/100					3	12

Table S5 Continued

Organism(s) with highest similarity (GenBank Accession number) classified according to lineage	Similarity (%)	A <sub>D</sub>		B <sub>T2</sub>		C <sub>D</sub>	
		d 118		d 41	d 138	d 60	
		glass	CPVC	glass	glass	glass	CPVC
<b><i>Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae</i></b>							
Uncultured <i>Rhodocyclaceae</i> bacterium clone fjc-40 16S ribosomal RNA gene, partial sequence (JQ278785)/ Uncultured bacterium clone BF-17 16S ribosomal RNA gene, partial sequence (HQ609626)	95/95				1		
<i>Delftia acidovorans</i> strain 7K11A 16S ribosomal RNA gene, partial sequence (KT825833)/ <i>Delftia</i> sp. ALBL_099 16S ribosomal RNA gene, partial sequence (KT865709)	99/99	1					
<b><i>Proteobacteria, Betaproteobacteria, Burkholderiales, Ralstonia</i></b>							
<i>Ralstonia</i> sp. OV225 16S ribosomal RNA gene, partial sequence (AY216797)/ Uncultured <i>Ralstonia</i> sp. partial 16S rRNA gene, clone Met_OH_55_5.3 (LN624415)	100/100	2					
<b><i>Proteobacteria, Betaproteobacteria, Nitrosomonadales, Nitrosomonadaceae</i></b>							
Uncultured prokaryote clone 16S ribosomal RNA gene, partial sequence (KF680693)/ Uncultured bacterium clone 5A-27 16S ribosomal RNA gene, partial sequence (JQ923569).	100/100				2	3	
Uncultured bacterium RNA for 16S rRNA, partial sequence, clone: 0423R003_K06 (AB656851)/ Uncultured prokaryote clone 16S ribosomal RNA gene, partial sequence (KF680693)	99/97				3		
Uncultured prokaryote clone 16S ribosomal RNA gene, partial sequence (KF680693)/ Uncultured bacterium clone 5A-27 16S ribosomal RNA gene, partial sequence (JQ923569)	97/97					1	2
Uncultured prokaryote clone 16S ribosomal RNA gene, partial sequence (KF680693)	98						1
Uncultured prokaryote clone 16S ribosomal RNA gene, partial sequence (KF680693)	99						1



Table S5 Continued

Organism(s) with highest similarity (GenBank Accession number) classified according to lineage	Similarity (%)	A <sub>D</sub>		B <sub>T2</sub>		C <sub>D</sub>	
		d 118		d 41	d 138	d 60	
		glass	CPVC	glass	glass	glass	CPVC
<b><i>Proteobacteria, Betaproteobacteria, Rhodocylales, Rhodocyclaceae</i></b>							
<i>Methyloversatilis discipulorum</i> strain FAM1 16S ribosomal RNA, complete sequence (NR_136517)/ <i>Methyloversatilis universalis</i> strain FAM5 16S ribosomal RNA gene, partial sequence (NR_043813)	100/99			9		3	14
<i>Methyloversatilis discipulorum</i> strain FAM1 16S ribosomal RNA, complete sequence (NR_136517)	97			1		1	
Uncultured <i>Rhodocyclaceae</i> bacterium clone hmx-283 16S ribosomal RNA gene, partial sequence (JQ278885)/ Uncultured <i>Azospira</i> sp. clone fjc-44 16S ribosomal RNA gene, partial sequence (JQ278789)	99/99			4	3	17	2
Uncultured <i>Rhodocyclaceae</i> bacterium clone hmx-283 16S ribosomal RNA gene, partial sequence (JQ278885).	96			1			
<b><i>Proteobacteria, Betaproteobacteria, TRA3-20</i></b>							
Uncultured bacterium DSSD90 16S ribosomal RNA gene, partial sequence (AY328787)/Uncultured bacterium clone Reactor4_47 16S ribosomal RNA gene, partial sequence (JQ629783)	100/100			4	1		
<b><i>Proteobacteria: Gammaproteobacteria</i></b>							
<i>Legionella pneumophila</i> 2300/99 Alcoy, complete genome (CP001828)/ <i>Legionella pneumophila</i> str. Paris complete genome (CR628336)	100					1	
<i>Moraxellaceae</i> bacterium W2.09-231 16S ribosomal RNA gene, partial sequence (JX458449)	97		8				
<i>Xanthomonadaceae</i> bacterium L-bf-PMW-15.1 partial 16S rRNA gene, strain L-bf-PMW-15.1 (FR774560)	99		1				

Table S5 Continued

Organism(s) with highest similarity (GenBank Accession number) classified according to lineage	Similarity (%)	A <sub>D</sub>		B <sub>T</sub> 2		C <sub>D</sub>	
		d 118		d 41	d 138	d 60	
		glass	CPVC	glass	glass	glass	CPVC
<b><i>Proteobacteria: Deltaproteobacteria</i></b>							
Uncultured bacterium clone LIB115_090_P1 16S ribosomal RNA gene, partial sequence (JX087323)/ Uncultured bacterium clone kab181 16S ribosomal RNA gene, partial sequence (FJ936898)	99/99		2				
<b><i>Acidobacteria, subgroup 4</i></b>							
<i>Chloracidobacterium</i> sp. enrichment culture clone JAC25 16S ribosomal RNA gene, partial sequence (KC161245)/ Uncultured <i>Acidobacteria</i> bacterium partial 16S rRNA gene, isolate OTU9/APA (AM902634)	100/99	11	7			5	7
<i>Chloracidobacterium</i> sp. enrichment culture clone JAC25 16S ribosomal RNA gene, partial sequence (KC161245)	98						1
Uncultured <i>Acidobacteria</i> bacterium partial 16S rRNA gene, isolate OTU9/APA (AM902634)	92					1	
<b><i>Bacteroidetes: Cytophagia, Cytophagales, Cytophagaceae</i></b>							
Uncultured <i>Cytophaga</i> sp. clone 166 16S ribosomal RNA gene, partial sequence (GU245900)	94		1				
<b><i>Gemmatimonadetes: Gemmatimonadales</i></b>							
Uncultured bacterium clone HDB_SIOU403 16S ribosomal RNA gene, partial sequence (HM186700)	100		2				1
Uncultured bacterium clone 109109H07 16S ribosomal RNA gene, partial sequence (JN200249)	99			2	4		
Uncultured soil bacterium clone L1A.1D03 16S ribosomal RNA gene, partial sequence (AY988636).	99				1		
<b><i>Planctomycetes</i></b>							
Uncultured <i>Planctomycetaceae</i> bacterium clone 44 16S ribosomal RNA gene, partial sequence (HQ462508)	97				3		
Total number of identified sequences		43	46	46	42	45	46

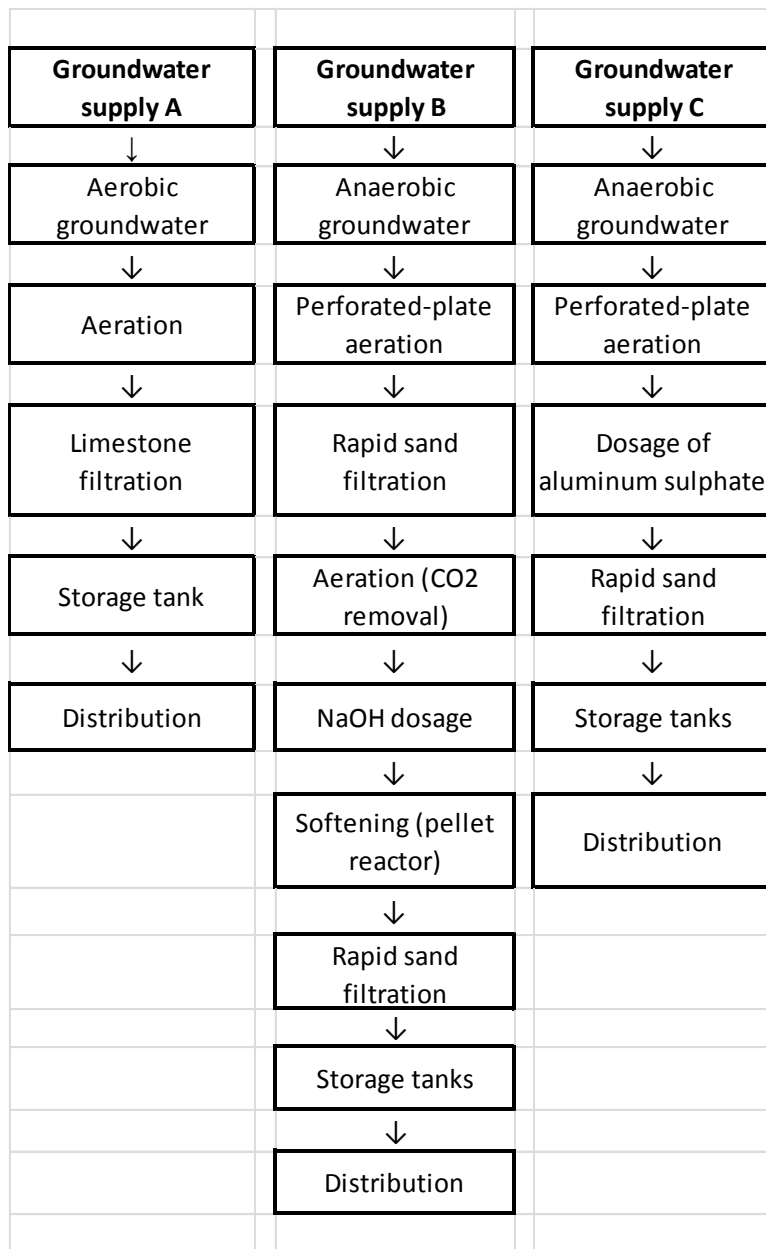


Fig. S1 Treatment schemes of groundwater supplies A, B and C.



Fig. S2. Time line of the investigations with the BBM system. Test series no. 1: water supply C, distribution system location ( $C_D$ ); no.2: water supply A, finished water ( $A_T$ ); no.3: water supply B, finished water ( $B_{T1}$ ); no.4: water supply B, finished water ( $B_{T2}$ ); no.5: water supply B, distribution system ( $B_D$ ); no.6: water supply A, distribution system ( $A_D$ ).

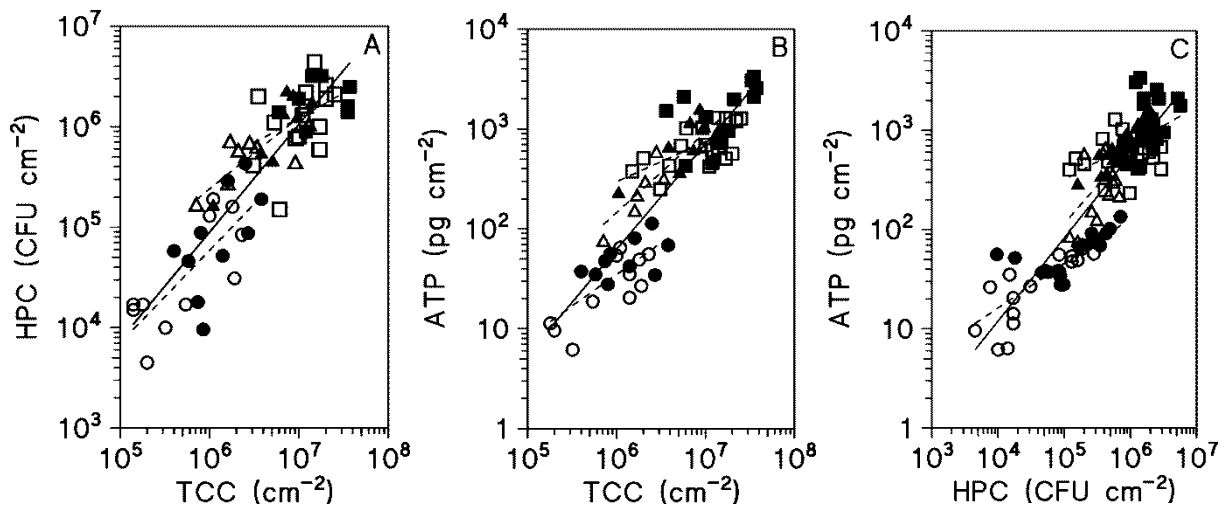


Figure S3. Relationships between total cell count (TCC) and heterotrophic plate count (HPC) (A), TCC and adenosine triphosphate (ATP) (B), and HPC-ATP (C) in the biofilms on glass and CPVC in the BBM system at water types A, B and C. Broken lines show relationship at individual water type. Symbols: ○ glass, supply A; ● CPVC, supply A; □ glass, supply B; ■ CPVC, supply B; △ glass, supply C and ▲ CPVC, supply C.

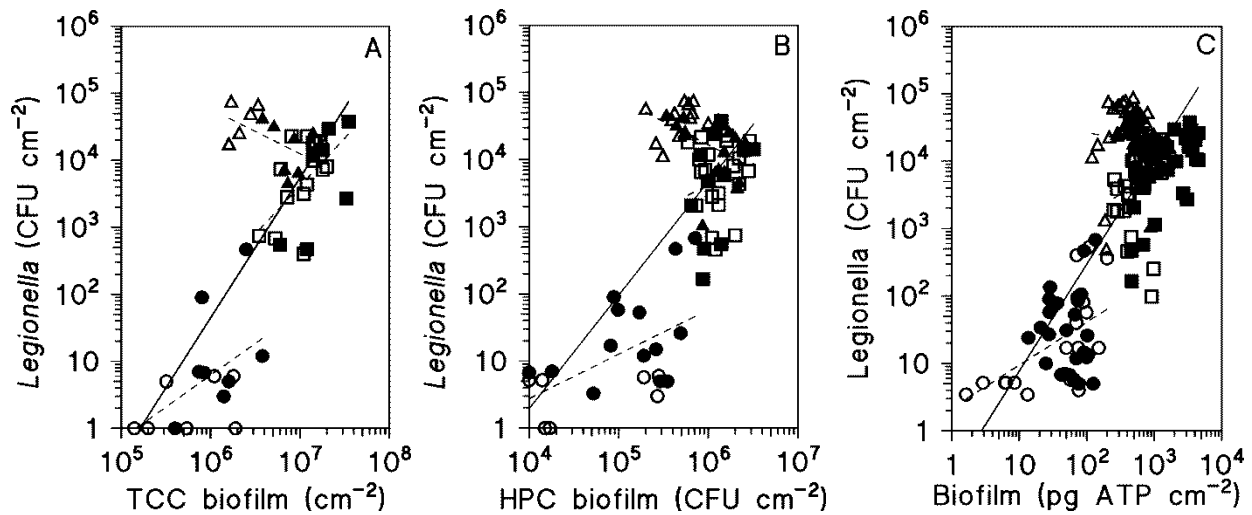


Figure S4. Relationships between *Legionella* colony count and TCC (A), HPC (B) and ATP (C) in the biofilms on glass and CPVC in the BBM system at water types A, B and C. Broken lines show relationship at individual water type. For symbols, see the legend to Fig. S3.

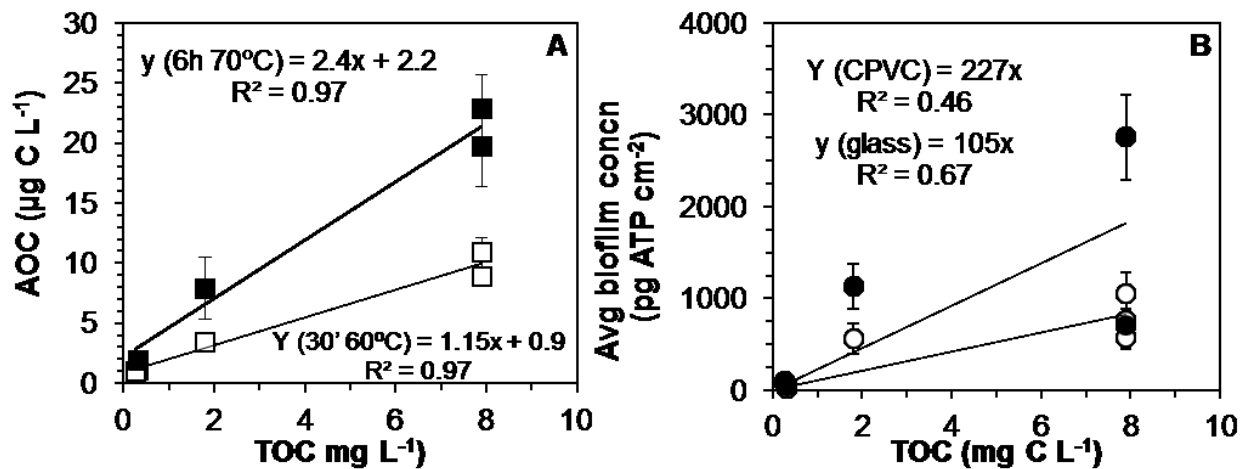


Figure S5. A: Relationship between the concentration of AOC and TOC. Symbols: □ standard AOC (30 min 60 °C), ■ AOC 6 h 70 °C; B: Relationship between the average biofilm concentration on glass (○) and the TOC concentration ( $P=0.03$ ) and the average biofilm concentration on CPVC (●) and the TOC concentration ( $P=0.19$ ).