Sewer biofilm microbiome and antibiotic resistance genes as function of pipe material, source of microbes, and disinfection: field and laboratory studies

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Supplemental Information

Targeted antibiotic resistance gene rationale

The targeted antibiotic resistance genes (ARGs) were chosen to provide a range of resistance mechanisms and vary in importance to human health. Sulfonamide antibiotics competitively inhibit the action of dihydropteroate synthase (DHPS), an intermediate step in folate synthesis which is a vital compound for nucleic acid synthesis in some bacteria.[1] *sul*1 encodes for an alternative DHPS in Gram-negative bacteria, which has a low affinity for sulfonamide antibiotics, thus conferring resistance.[2] Functional bla_{TEM} genes are mostly found in enterobacteria and produce a β-lactamase that hydrolyses the cyclic amide bond in β-lactam antibiotics.[3] *tet* genes confer multiple mechanisms of resistance to tetracycline antibiotics which target bacterial ribosomes and inhibit protein synthesis. *tet(*G) encodes for an efflux pump specific for tetracycline that pumps the drug out of the cell of Gram-negative bacteria.[4] *tet*(O) and *tet*(W) encode for proteins that interfere with the binding of tetracycline to the ribosome, thus providing ribosomal protection against the drug.[4] *erm*F encodes for an erythromycin ribosomal methylase, an enzyme that modifies the 50S ribosomal subunit which is the target site of erythromycin antibiotics.[5] New Delhi metallo-β-lactamase (NDM-1) is a recently discovered metallo-β-lactamase ARG mainly found in Gram-negative bacteria, that confers resistance to most β-lactam antibiotics including carbapenems, by hydrolyzing the β-lactam ring of the drug.[6, 7] Other ARGs of interest such as *van*A (vancomycin resistance gene), *mex*B (multidrug resistance gene), *qnr* (quinolone resistance gene) and *mec*A (methicillin resistance gene) resulted in non-specific binding following optimization efforts including temperature gradients and Mastermix adjustments in this matrix. This may indicate these genes were not present in this matrix as the primer sets used have been used by us and others successfully on other matrices.

Alpha Diversity: 3500 sequence depth vs. no subsampling (rarefaction)

Results of the Shannon and Simpson alpha diversity indices at a depth of 3500 sequences showed that field biofilm samples had similar diversity compared to the biofilm communities grown in both experiments ($p > 0.072$, Kruskal–Wallis test). However, Sed-SB biofilms had significantly greater prokaryotic diversity indices (Shannon and Simpson) and richness than WW-CF biofilms ($p < 0.041$, Kruskal–Wallis test; Table S3). Similar results were obtained when the data was analyzed without sequence subsampling (rarefaction), ($p < 0.034$, Kruskal–Wallis

test; Table S4A). Evenness was significantly higher in Sed-SB biofilms compared to biofilms from the field and WW-CF experiment in both the 3500-sequence subsample and with no subsampling ($p < 0.031$ and $p < 0.046$, Kruskal–Wallis test).

Control study of sonication and centrifugation

To confirm that our sample preparation (including the sonication and centrifugation) would not impact our viable-cell qPCR results, a control study was performed with swabbed *E. coli* collected at exponential growth. There was no significant difference between the *E. coli* sample treated with PMA and total DNA of the same culture based on qPCR targeting the 16S rRNA gene (p=0.25, Fig. S8) indicating that the sonication method did not impair cell integrity enough to impact our results by causing an underestimation of viable cell gene copies.

Microbiome analysis and biomarkers determined by LEfSe

Taxonomical microbial diversity analysis showed that each biofilm sample type (Field, Sed-SB, WW-CF) was dominated by different family taxa. The families observed included those containing potential human pathogens, obligate anerobic hydrogenotrophs known to cause corrosion, and nitrogen oxidizing bacteria (Fig. S6 and S7). To identify biomarkers associated with the different biofilm samples, LEfSe was performed. No prokaryote biomarkers were found as a factor of pipe material or between field sample location (System 1 vs System 2). However, 27 biomarkers were detected as a function of biofilm sample type (Field, Sed-SB, WW-CF biofilm samples; Fig. S8). Results indicated that some of the 16S rRNA gene signatures detected are affiliated within taxonomic orders encompassing sulfate reducing bacteria like Desulfovibrionales and Desulfobacterales, which are known to be involved in microbiological influenced corrosion (MIC).[8] In addition, Campylobacterales which is the taxonomical order of the known pathogen *Campylobacter jejuni* was significantly more abundant ($p < 0.05$) in Sed-SB biofilm. In WW-CF biofilm, methanogenic Archaea (Methanomicrobiales) which also play a role in MIC[8] were detected as biomarkers, as well as Chlamydiales which contains obligate intracellular pathogenic species.[9] In biofilms sampled from field municipal sewers, Nitrospira which is a taxonomical class containing bacteria involved in nitrogen oxidation and are commonly found in all WWTP,[10] was significantly more abundant. In addition, the order Legionellales, that includes species of human pathogen Legionella pneumophila was a biomarker for field sewer biofilm. Taxonomic analysis to the species level did not showed the presence of pathogenic species belonging to these taxa, however, longer sequence reads are necessary to confirm the presence or absence of bacterial species.

Table S1. (A) Information about the municipal sewer systems where samples were collected. (B) Water quality measurements of the wastewater used to feed the reactor. (C) pH and conductivity measurements of the sediment used to feed Sed-SB reactor.

A **Sampled municipal sewer systems information**

System ID	Sewer Type	Field Biofilm sampling	Field Sediment sampling (Sed-SB)	Wastewater source (WW-CF)
System A	Combined		X	
System 1	Separate	X	X	
System 2	Separate	X		X

B **Wastewater abiotic factors**

Reactor feed	TSS (mg/L)	COD (mg/L)	pH	Conductivity (µS/cm)
Sed-SB (1st reactor run)	316.7±17	562±12	7.5±0.2	470±41
Sed-SB (2nd reactor run)	328.7±21	2714±62	6.6±0.2	1262±68
WW-CF	223.3±14	317±23	7.4±0.1	322.2±26

C **Abiotic factors of sediment used for Sed-SB rector setup**

System ID	pH	Conductivity (uS/cm)
System 1 (1st reactor run)	7.88	97.91
System A (1st reactor run)	7.7	59.33
System 1 (2nd reactor run)	5.72	1242
System A (2nd reactor run)	6.63	316.1

Table S2 Primers, PCR conditions temperatures, and amplicon lengths. For PCR conditions "m" means time in minutes and "s" means time in seconds.

Gene	Primer sequence (5'-3')	PCR conditions	Amplicon length (bp)	R^2/Efficiency	Source
*sul*1	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	95°C for 2m [98°C for 5s, 69.9°C for 5s]^{x40}	163	0.99±0.1/97±4%	11
tet(G)	GCAGAGCAGGTCGCTGG CCYGCAAGAGAAGCCAGAAG	98°C for 2m [98°C for 5s, 64°C for 5s]^{x40}	134	0.99±0.1/95±5%	12
ermF	CGACACAGCTTTGGTTGAAC GGACCTACCTCATAGACAAG	95°C for 4m [94°C for 30s, 56°C for 30s and 72°C for 30s]^{x40}	309	0.98±0.1/94±5%	13
bla$_{TEM}$	TTCCTGTTTTTGCTCACCCAG CTCAAGGATCTTACCGCTGTTG	95°C for 3m [95°C for 15s, 60°C for 20s]^{x40}	247	0.98±0.2/92±5%	14
tet(O)	ACGGARAGTTTATTGTATACC TGGCGTATCTATAATGTTGAC	98°C for 2m [98°C for 5s, 50°C for 5s]^{x40}	171	0.99±0.1/95±4%	15
tet(W)	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	98°C for 2m [98°C for 5s, 60°C for 5s]^{x40}	168	0.98±0.1/94±6%	15
NDM-1	TTTCAGTCCGACACAACGCG CAGCCACCAAAAGCGATGTC 6-FAM-CAACCGCGCCCAACTTTGGC-TAMRA	98°C for 15m [98°C for 30s, 59°C for 1m]^{x40}	155	0.97±0.2/89±6%	6
HF183	ATCATGAGTTCACATGTCCG TACCCCGCCTACTATCTAATG	98°C for 10m [95°C for 15s, 53°C for 30s]^{x40}	82	0.99±0.1/94±6%	16
16S rRNA	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	95°C for 10m [95°C for 15s, 60°C for 1m]^{x40}	202	0.97±0.2/93±6%	17

Table S3 Indices and measurements of microbial alpha-diversity for field and reactor biofilm samples at a 3500 sequences subsample depth.

Sample	Biofilm from:	Shannon	Richness	Evenness
System 1 (A)	Field	1.74	28	0.52
System 1 (B)	Field	2.94	100	0.64
System 1 (C)	Field	3.16	122	0.66
System 1 (D)	Field	2.49	83	0.56
System 2 (A)	Field	2.72	80	0.62
System 2 (B)	Field	2.99	86	0.67
System 2 (C)	Field	3.88	162	0.76
System 2 (D)	Field	3.71	148	0.74
Reactor PVC – WW-CF (A)	Wastewater	2.22	47	0.58
Reactor PVC – WW-CF (B)	Wastewater	2.15	42	0.58
Reactor Concrete – WW-CF (A)	Wastewater	2.43	54	0.61
Reactor Concrete – WW-CF (B)	Wastewater	2.46	54	0.62
Reactor PVC – Sed-SB (A)	Sediment	3.51	116	0.74
Reactor PVC - Sed-SB (B)	Sediment	3.49	83	0.79
Reactor Concrete - Sed-SB (A)	Sediment	3.62	110	0.77
Reactor Concrete - Sed-SB (B)	Sediment	3.16	69	0.75

Table S4. Indices and measurements of microbial alpha-diversity of field and reactor biofilm samples with no rarefaction. (A) Represent the samples from the field and biofilm growth in the reactor, and (B) the samples from the disinfection experiment. (Note letters A-D following the sample names are used to indicate different replicates).

A.

Sample	Biofilm from:	Shannon	Richness	Evenness
System 1 (A)	Field	1.45	49	0.37
System 1 (B)	Field	2.68	123	0.56
System 1 (C)	Field	2.99	138	0.61
System 1 (D)	Field	2.29	115	0.48
System 2 (A)	Field	2.66	99	0.58
System 2 (B)	Field	2.99	118	0.63
System 2 (C)	Field	3.68	186	0.70
System 2 (D)	Field	3.57	183	0.69
Reactor PVC - WW (A)	Wastewater	1.99	52	0.50
Reactor PVC - WW (B)	Wastewater	1.89	45	0.50
Reactor Concrete - WW (A)	Wastewater	2.40	56	0.60
Reactor Concrete - WW (B)	Wastewater	2.43	57	0.60
Reactor PVC - SED (A)	Sediment	4.37	323	0.76
Reactor PVC - SED (B)	Sediment	3.18	84	0.72
Reactor Concrete - SED (A)	Sediment	4.32	343	0.74
Reactor Concrete - SED (B)	Sediment	2.87	69	0.68

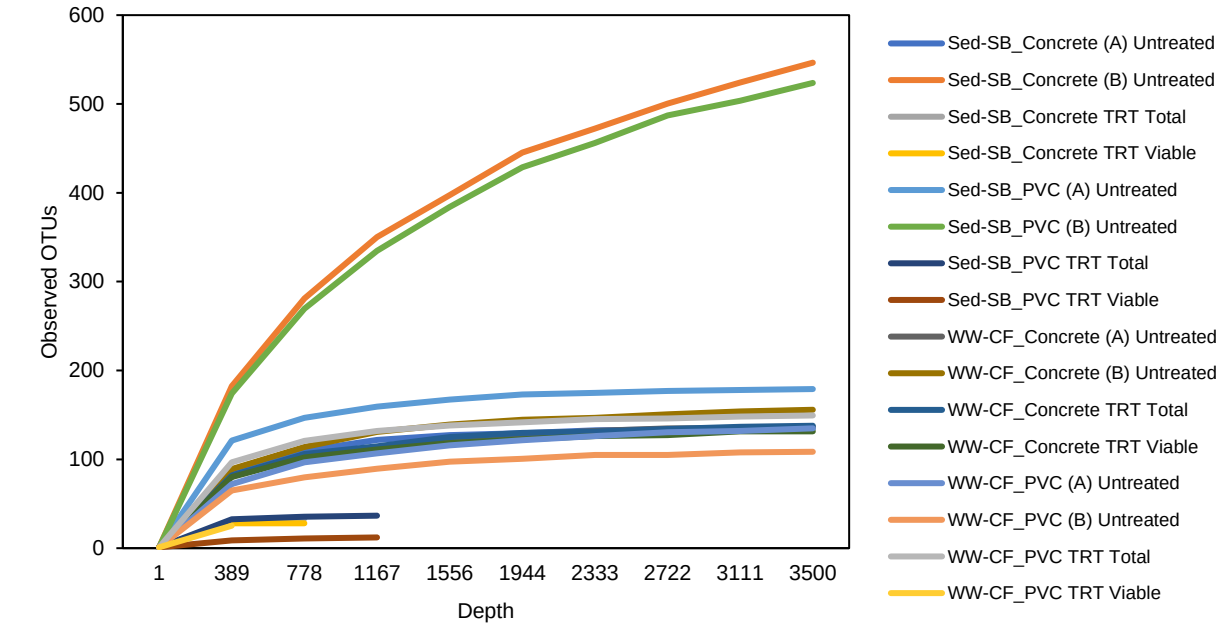
B.

Biofilm from:	Surface	Treatment	Shannon	Richness	Evenness
Wastewater	PVC	Treated (Total)	3.01	65	0.72
Wastewater	PVC	Treated (Viable)	2.07	20	0.69
Wastewater	Concrete	Treated (Total)	2.47	58	0.61
Wastewater	Concrete	Treated (Viable)	2.40	50	0.61
Sediment	PVC	Treated (Total)	2.41	26	0.74
Sediment	PVC	Treated (Viable)	0.30	9	0.14
Sediment	Concrete	Treated (Total)	2.54	20	0.85
Sediment	Concrete	Treated (Viable)	2.11	19	0.72

Table S5 Indices and measurements of microbial alpha-diversity of the reactor's biofilm samples before and after the bleach treatment. Measurements were calculated at a 500 sequences subsample depth (rarefaction)

Biofilm from:	Surface	Treatment	Shannon	Simpson	Inverse Simpson	Fisher	Richness	Evenness
Sediment	Concrete	Untreated	3.11	0.92	12.8	14.2	51	0.79
Sediment	Concrete	Untreated	3.73	0.95	22.0	29.9	86	0.84
Sediment	Concrete	Treated (Total)	2.56	0.90	10.0	4.17	20	0.85
Sediment	Concrete	Treated (Viable)	2.25	0.85	6.79	3.40	17	0.79
Sediment	PVC	Untreated	3.45	0.95	19.7	17.0	83	0.79
Sediment	PVC	Untreated	3.51	0.94	15.5	28.4	58	0.85
Sediment	PVC	Treated (Total)	2.81	0.91	11.6	5.82	26	0.86
Sediment	PVC	Treated (Viable)	0.40	0.15	1.17	1.35	8	0.19
Wastewater	Concrete	Untreated	2.35	0.84	6.14	9.22	37	0.65
Wastewater	Concrete	Untreated	2.37	0.83	6.03	8.57	35	0.67
Wastewater	Concrete	Treated (Total)	2.51	0.85	6.64	9.22	37	0.70
Wastewater	Concrete	Treated (Viable)	2.66	0.87	7.58	9.22	37	0.74
Wastewater	PVC	Untreated	2.12	0.80	5.01	6.11	27	0.64
Wastewater	PVC	Untreated	2.34	0.83	5.72	6.89	39	0.77
Wastewater	PVC	Treated (Total)	2.17	0.81	5.31	6.41	28	0.65
Wastewater	PVC	Treated (Viable)	2.31	0.84	6.28	4.17	20	0.61

A.



B.

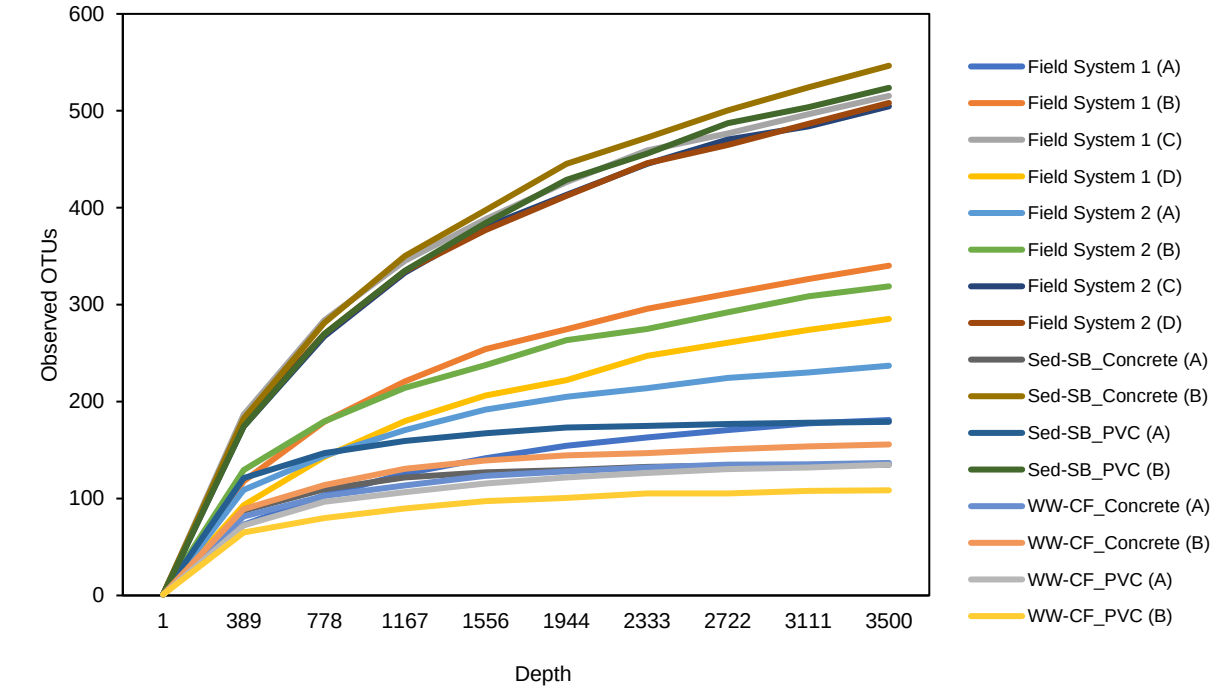


Fig. S1. Rarefaction curves for samples of both (A) disinfection experiment and (B) biofilm growth, and field biofilm samples. The plots represent the comparison of the observed OTUs (y axis) and the sequences depth (x axis).

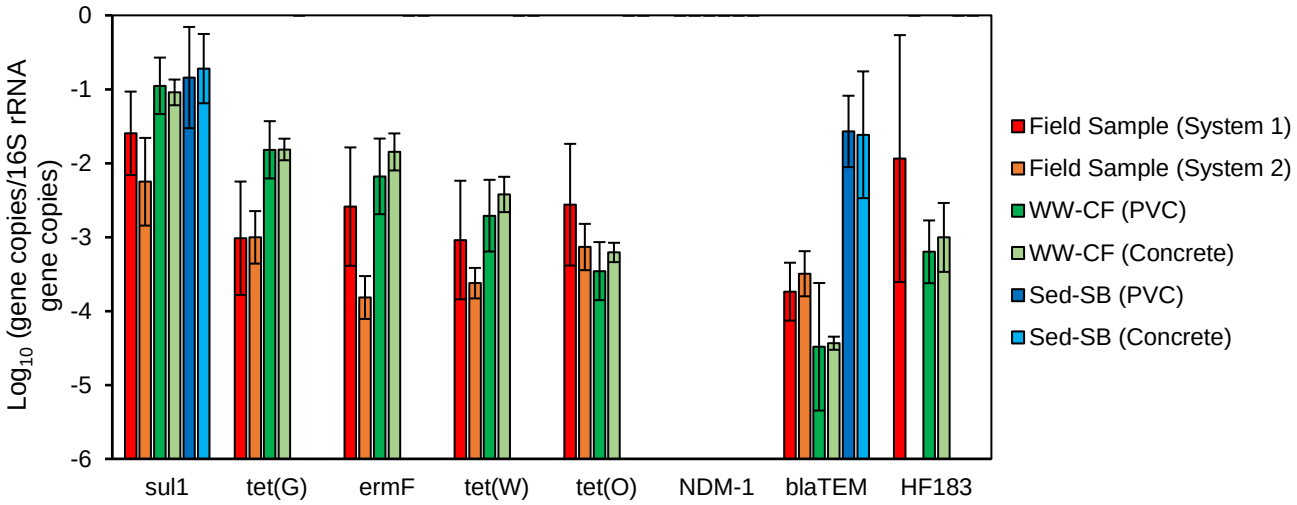


Fig. S2 Relative abundance of ARGs and human fecal indicator HF183 (i.e., normalized to 16S rRNA gene copies) for field biofilm samples (2 sites) and the simulated systems (WW-CF and Sed-SB) in both pipe materials (PVC and concrete). Error bars represent the standard deviation of the mean (*n*=6 for "System 1", *n*=5 for "System 2" and *n*=4 for all the biofilm reactor samples).

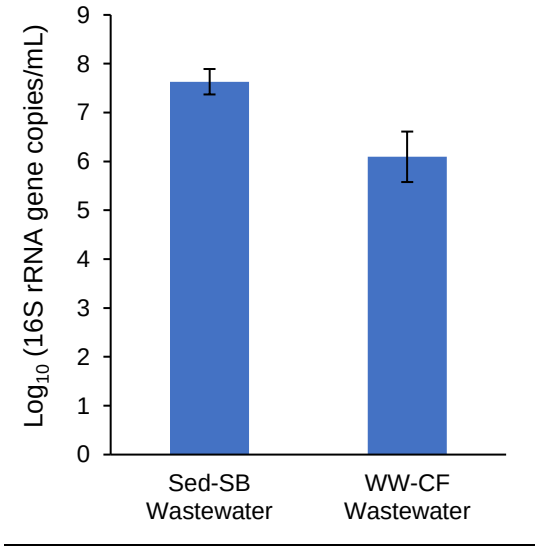


Fig. S3 Comparison of the concentration of 16S rRNA gene copies between the wastewater used to feed each reactor setup (Sed-SB vs WW-CF). Error bars represent the standard deviation of the mean (*n*=2 for Sed-SB and *n*=3 for WW-CF)

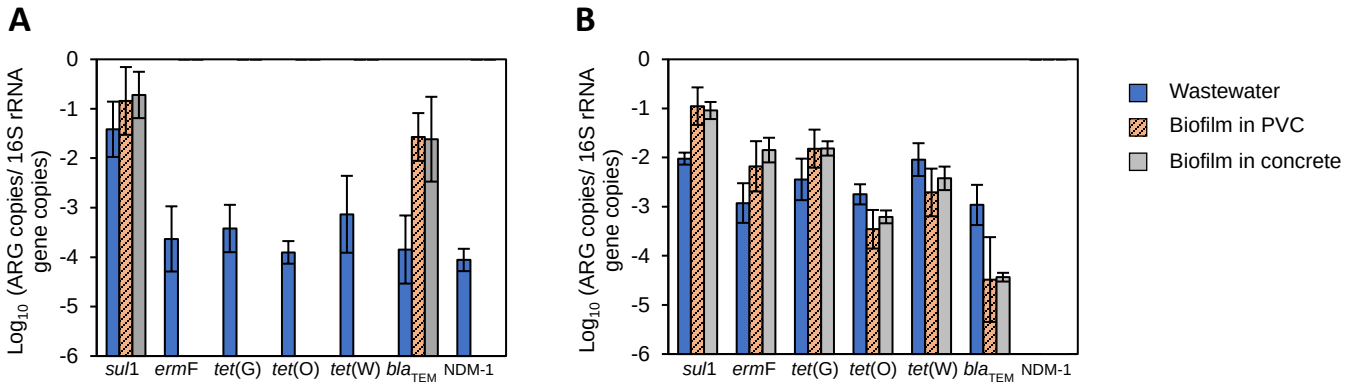


Fig. S4 Relative abundance of ARGs (i.e., normalized to 16S rRNA gene copies) for feed wastewater and the biofilm for reactor **(A)** Sed-SB and **(B)** WW-CF. In the Sed-SB plot (A) $n = 4$ in all the samples while in WW-CF plot (B) $n = 3$ for wastewater and $n = 4$ for each biofilm sample (on PVC and on concrete). Error bars represent the standard deviation of the mean.

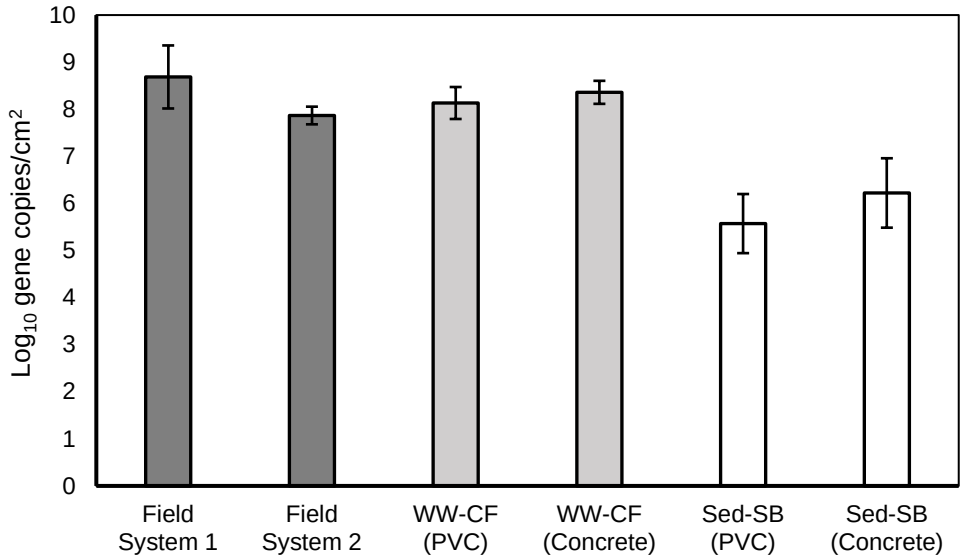


Fig. S5 Concentration of 16S rRNA gene copies per area for the field biofilm samples (2 sites) and the reactor biofilm in both setups (WW-CF and Sed-SB) and pipe materials (PVC and Concrete). Error bars represent the standard deviation of the mean (*n*=6 for field biofilm samples and *n*=4 for all the biofilm reactor samples).

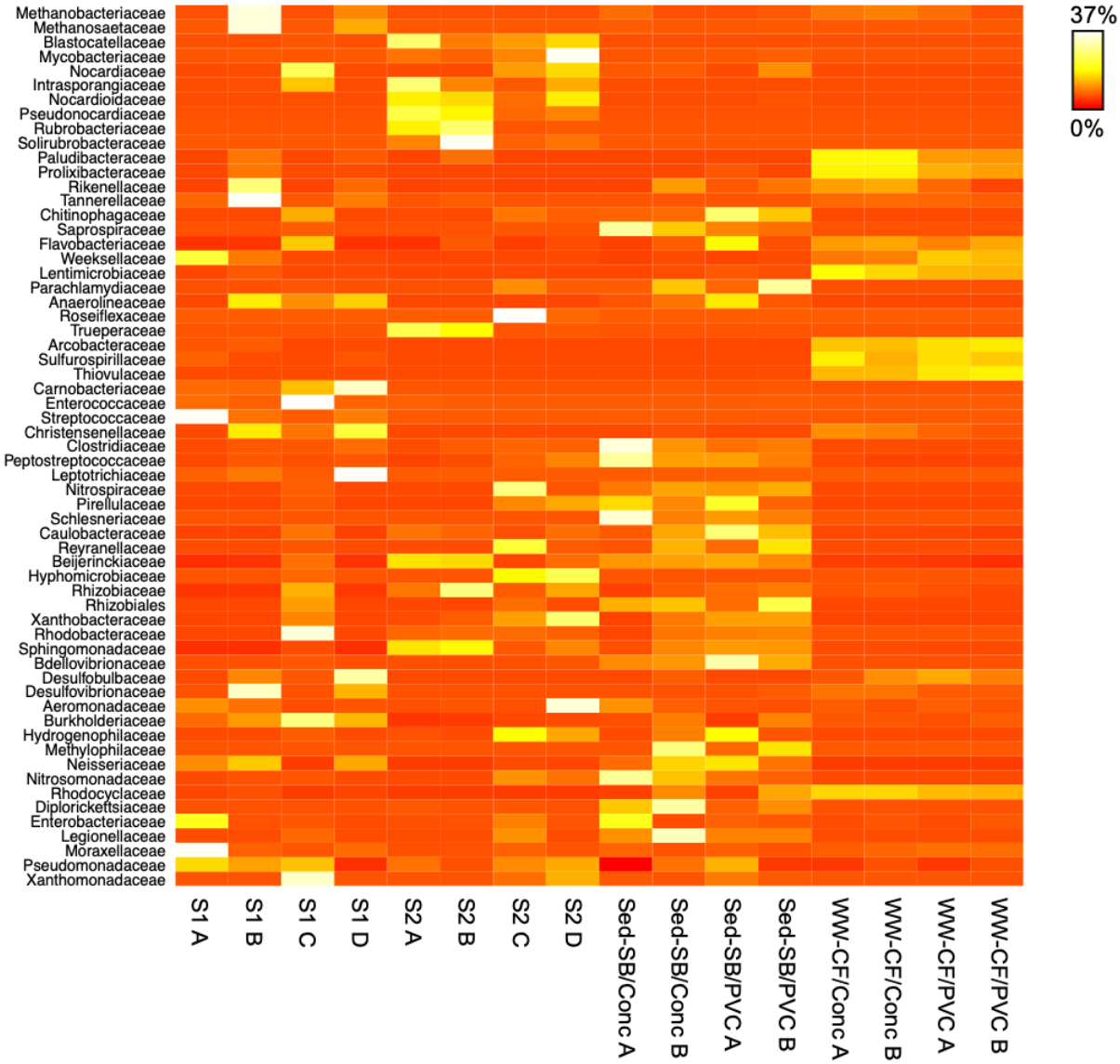


Fig S6. Heatmap showing the microbiome at the family level of all field and reactor biofilm samples. White color represents 37% relative abundance of the taxon, while red means 0% relative abundance. Heatmap was created with a 3500-sequence subsample (rarefaction).

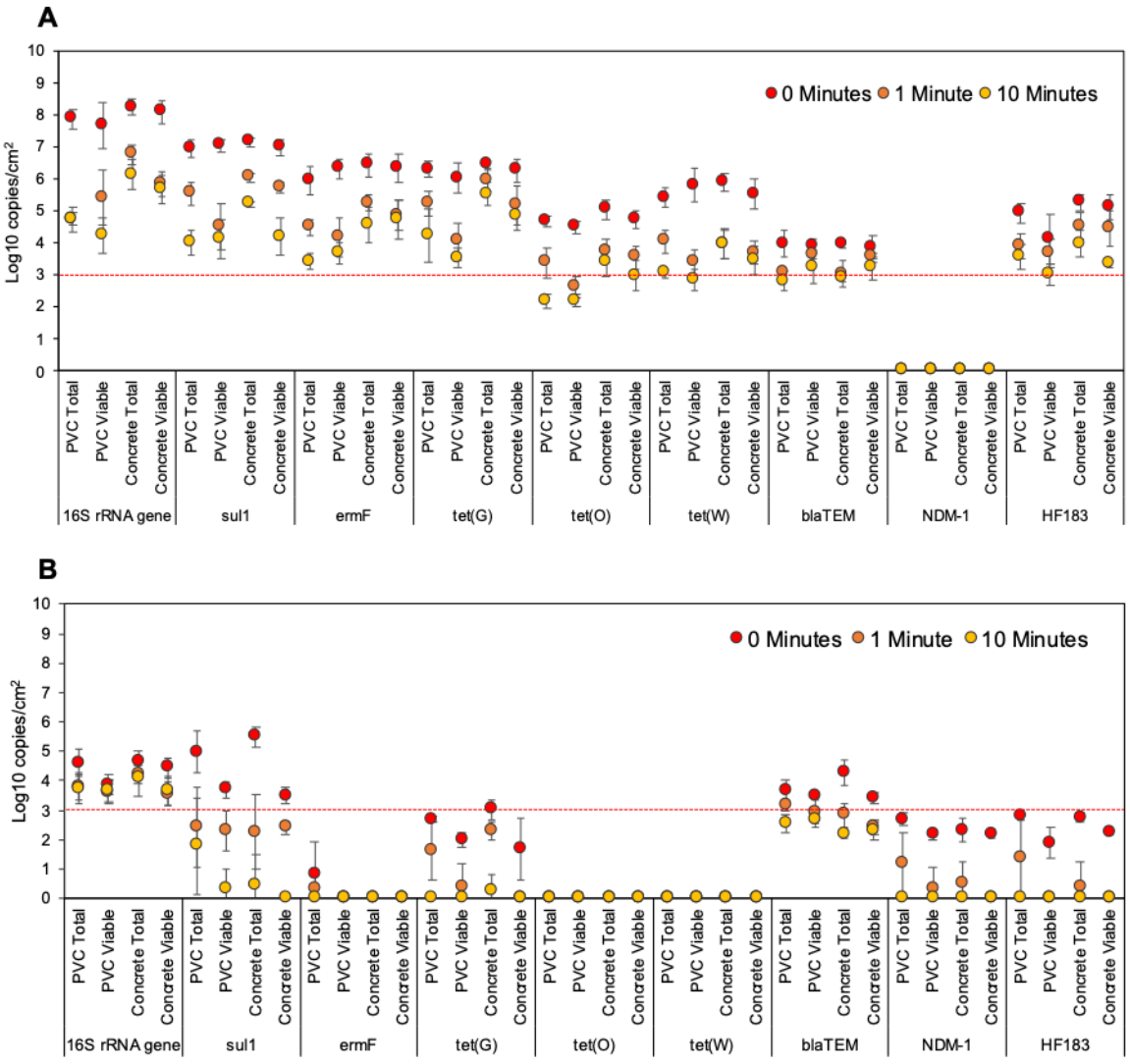


Fig. S7. Concentrations of total and viable cells carrying viable 16S rRNA gene and ARG copies in the biofilms of both reactor setups **(A)** WW-CF and **(B)** Sed-SB before the treatment with bleach (0 minutes; red circle), after 1 minute of exposure to the disinfectant (orange square) and 10 minutes of exposure (yellow triangle). *n*=4 for all WW-CF samples and *n*=5 for all Sed-SB samples. Error bars represent the standard deviation of the mean. The red dotted line represents the LOQ based on the lowest value in the qPCR standard curve.

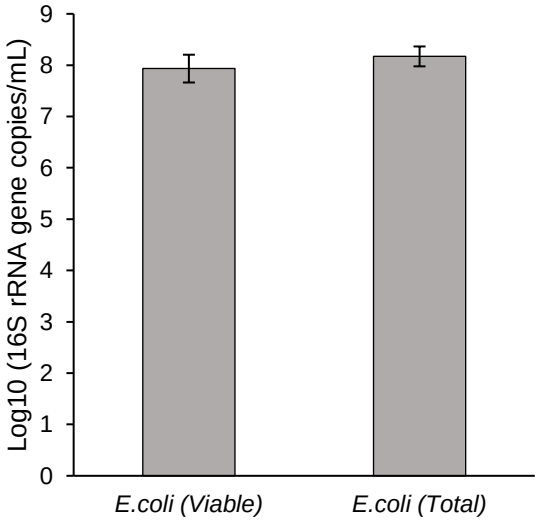


Fig. S8 Concentrations of the total and viable-cells carrying 16S rRNA gene. Samples were collected from swabs of a culture of *Escherichia coli* (TOP10) and analyzed following dislodging by sonication. Error bars represent the standard deviation of technical replicates (*n*=3).

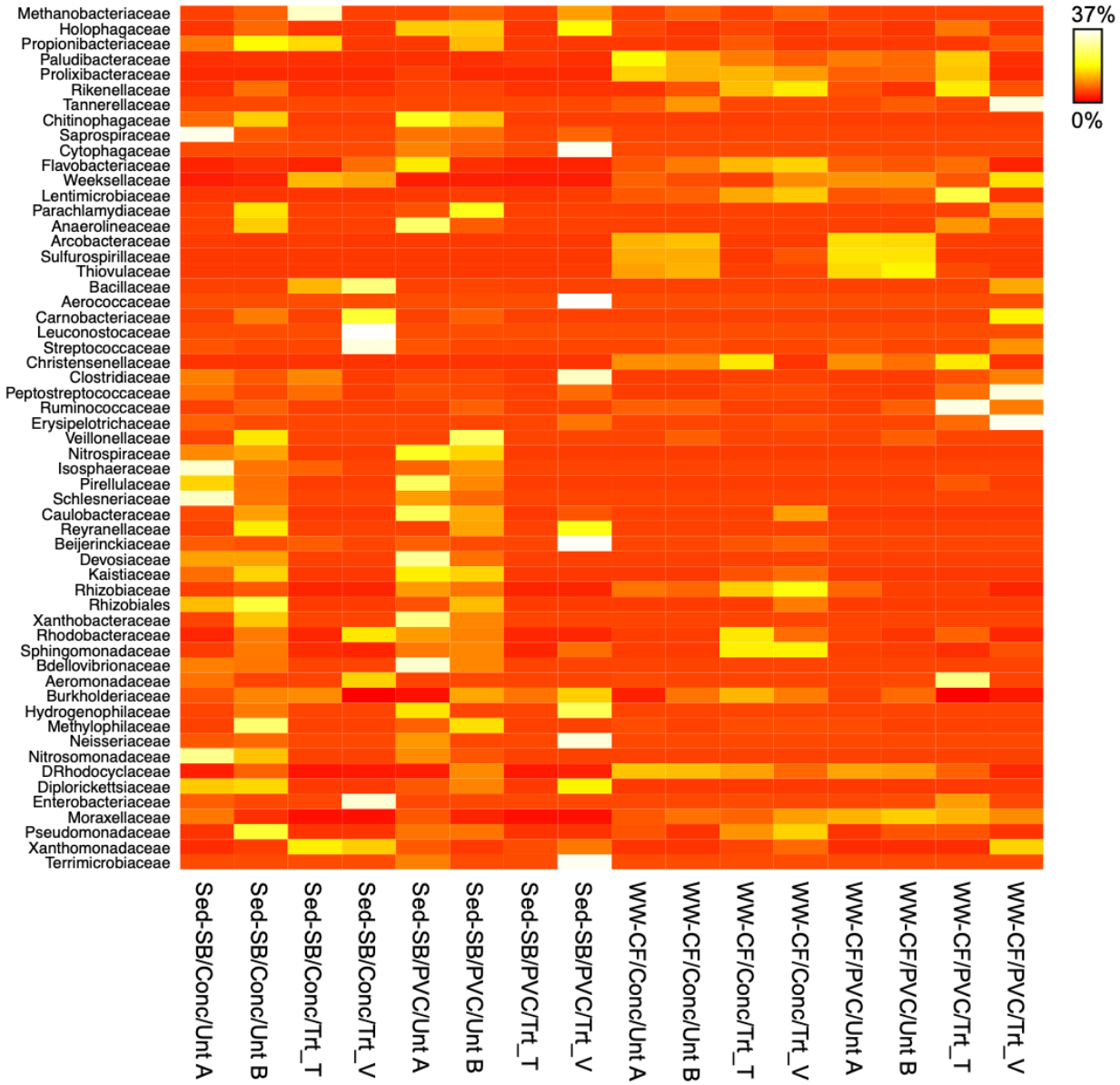


Fig. S9. Heatmap of the biofilm microbiome in both reactor setups (Sed-SB and WW-CF) at the family level before (Unt) and after (Trt)10 minutes of exposure to 4.6% bleach for total (T) DNA and DNA from viable (V) cells. White color represents 37% relative abundance of the taxon while red represents 0% relative abundance. The heatmap was created with a 500-sequences subsample depth (rarefaction).

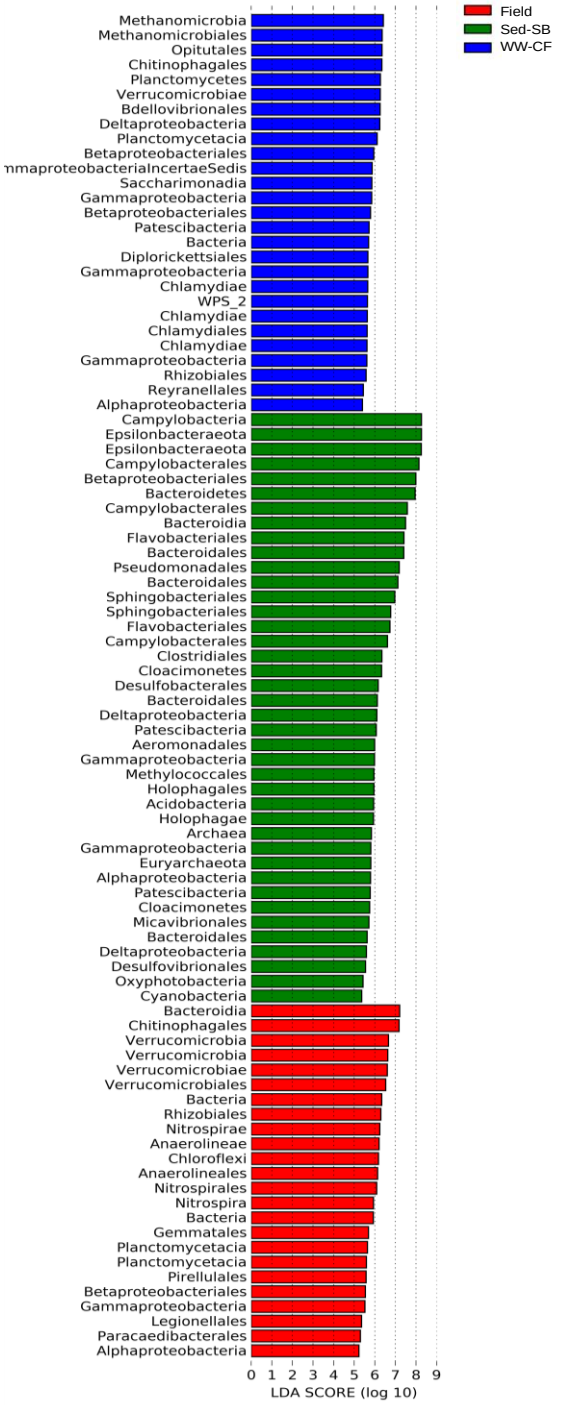


Fig. S10. Linear discriminant analysis (LDA) effect size (LEfSe) histogram. LDA Score of the most abundant bacteria in the different types of biofilm samples

References

1. Skold, O., Sulfonamide resistance: mechanisms and trends. *Drug Resist Updat* **2000,** *3* (3), 155-160.
2. Jiang, H.; Cheng, H.; Liang, Y.; Yu, S.; Yu, T.; Fang, J.; Zhu, C., Diverse Mobile Genetic Elements and Conjugal Transferability of Sulfonamide Resistance Genes (sul1, sul2, and sul3) in Escherichia coli Isolates From Penaeus vannamei and Pork From Large Markets in Zhejiang, China. *Front Microbiol* **2019,** *10*, 1787.
3. Muhammad, I.; Golparian, D.; Dillon, J. A.; Johansson, A.; Ohnishi, M.; Sethi, S.; Chen, S. C.; Nakayama, S.; Sundqvist, M.; Bala, M.; Unemo, M., Characterisation of blaTEM genes and types of beta-lactamase plasmids in Neisseria gonorrhoeae - the prevalent and conserved blaTEM-135 has not recently evolved and existed in the Toronto plasmid from the origin. *BMC Infect Dis* **2014,** *14*, 454.
4. Gian Maria Rossolini, F. A., Tommaso Giani, Mechanisms of Antibacterial Resistance. In *Infectious Diseases*, Jonathan Cohen, W. G. P. a. S. M. O., Ed. Elsevier: 2017; Vol. 2, p 2016.
5. Xing, L.; Yu, H.; Qi, J.; Jiang, P.; Sun, B.; Cui, J.; Ou, C.; Chang, W.; Hu, Q., ErmF and ereD are responsible for erythromycin resistance in Riemerella anatipestifer. *PLoS One* **2015,** *10* (6), e0131078.
6. Diene, S. M.; Bruder, N.; Raoult, D.; Rolain, J. M., Real-time PCR assay allows detection of the New Delhi metallo-beta-lactamase (NDM-1)-encoding gene in France. *Int J Antimicrob Agents* **2011,** *37* (6), 544-6.
7. Yong, D.; Toleman, M. A.; Giske, C. G.; Cho, H. S.; Sundman, K.; Lee, K.; Walsh, T. R., Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. *Antimicrob Agents Chemother* **2009,** *53* (12), 5046-54.
8. Enning, D.; Garrelfs, J., Corrosion of iron by sulfate-reducing bacteria: new views of an old problem. *Appl Environ Microbiol* **2014,** *80* (4), 1226-36.
9. Borel, N.; Polkinghorne, A.; Pospischil, A., A Review on Chlamydial Diseases in Animals: Still a Challenge for Pathologists? *Vet Pathol* **2018,** *55* (3), 374-390.
10. Ushiki, N.; Fujitani, H.; Aoi, Y.; Tsuneda, S., Isolation of Nitrospira belonging to sublineage II from a wastewater treatment plant. *Microbes Environ* **2013,** *28* (3), 346-53.
11. Pei, R.; Kim, S. C.; Carlson, K. H.; Pruden, A., Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res* **2006,** *40* (12), 2427-35.
12. Aminov, R. I.; Chee-Sanford, J. C.; Garrigues, N.; Teferedegne, B.; Krapac, I. J.; White, B. A.; Mackie, R. I., Development, Validation, and Application of PCR Primers for Detection of Tetracycline Efflux Genes of Gram-Negative Bacteria. *Applied and Environmental Microbiology* **2002,** *68* (4), 1786-1793.
13. Chen, J.; Yu, Z.; Michel, F. C., Jr.; Wittum, T.; Morrison, M., Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Appl Environ Microbiol* **2007,** *73* (14), 4407-16.
14. Xi, C.; Zhang, Y.; Marrs, C. F.; Ye, W.; Simon, C.; Foxman, B.; Nriagu, J., Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Appl Environ Microbiol* **2009,** *75* (17), 5714-8.
15. Aminov, R. I.; Garrigues-Jeanjean, N.; Mackie, R. I., Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol* **2001,** *67* (1), 22-32.
16. Bernhard, A. E.; Field, K. G., A PCR assay To discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. *Appl Environ Microbiol* **2000,** *66* (10), 4571-4.
17. Muyzer, G.; De Waal, E. C.; Uitterlinden, A. G., Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis or polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **1993,** *59* (3), 6.