Electronic Supplementary Material (ESI) for Environmental Science: Water Research & Technology.

Supplementary Information

Permeate microbiome reflects the biofilm microbial community in a gravity-driven woven-fiber microfiltration (WFMF) system for wastewater treatment

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SI.1. Material and methods: Cell counts

The SYBR® Green I stain was prepared by diluting 10 µL of 10^4-concentrated SYBR® Green I stock solution in 990 µL of 10 mM Tris solution (Invitrogen) at pH 8. The SYBR® Green I and propidium iodide stain were prepared by mixing 10 µL of SYBR® Green I stock solution with 20 µL of 30 mM propidium iodide stock solution and 1 mL of 10 mM Tris at pH 8. Aliquots of both stains were stored at -20°C. The samples from the reactor and permeate were diluted 1:100 in deionized water. From the diluted samples, 500 µL were pipetted to a 3.5 mL tube, and 5 µL of the corresponding stain were added (i.e., SYBR® Green I for total cells and SYBR® Green I and propidium iodide for intact cells). Samples were dark incubated at 37°C for 10 min and then vortexed. A volume of 200 µL was transferred to a 96-deep-well plate and analyzed using the Beckman Coulter® cytometer (Beckman Coulter, Brea, CA, USA), equipped with a 50mW solid-state state laser emitting light at a fixed wavelength of 488 nm. Samples were measured in triplicates and conducted at a flow rate of 60 µL/min. Green fluorescence intensity (533 +/- 30 nm) and red fluorescence (> 670 nm) were measured on different channels. Results were evaluated using the software CytExpert (Beckman Coulter).

SI.2. Material and methods: Adenosine triphosphate (ATP)

The BacTiter-Glo™ reagent was prepared by mixing the BacTiter-Glo™ Buffer and BacTiter-Glo™. The BacTiter-Glo™ reagent was stored in aliquots of 58 µL (for aqueous samples) and 100 µL (for biofilm samples), in 2 mL Eppendorf tubes, at -20 °C for a maximum period of 7 days. The ATP assay protocol developed by Hammes et al. (2010) was followed step by step (1). To measure total ATP concentrations, 1 mL of unfiltered sample was pipetted into 2 mL Eppendorf tubes. To measure extracellular ATP concentrations, samples were filtered using a 5 mL syringe (Hauser) and a 0.2 µm syringe filter (Filtropur S 0.2 filter; Sarstedt, Nümbrecht, Germany), and 1 mL of the filtrate was collected in a 2 mL Eppendorf tube. Intracellular ATP concentrations were calculated as the difference between total and extracellular ATP concentrations. Filtered and unfiltered samples and the 58 µL BacTiter-Glo™ reagent aliquots (100 µL aliquots for biofilm samples) were incubated on a heating block at 38 °C for 4 min. While on the heating block, 750 µL of the sample (100 µL for biofilm samples) were pipetted into the reagent-containing tubes, incubated for precisely 20 seconds and immediately transferred to the luminometer to measure the luminescence in relative light units (RLU). A calibration curve was prepared using an ATP stock solution BacTiter-Glo™ Microbial Cell Viability Assay and following the steps in Hammes et al. (2010) to calculate the corresponding ATP concentration in nM (1).

SI.3. Materials and methods: 16S amplicon library construction, Illumina sequencing, and analysis of Illumina paired-end reads

Bacteria 16S V1-3 rRNA gene sequencing libraries were prepared by a custom protocol based on Caporaso et al. (2012) (2). Up to 10 ng of extracted DNA was used as the template for PCR amplification of the 16S V1-3 rRNA gene amplicons. Each PCR reaction (25 µL) contained dNTPs (100 µM of each), $MgSO_4$ (1.5 mM), Platinum Taq DNA polymerase HF (0.5 U/reaction), Platinum High Fidelity buffer (1X) (Thermo Fisher Scientific) and barcoded library adaptors (400 nM of each forward and reverse). PCR was conducted with the following program: Initial denaturation at 95 ^{o}C for 2 min, 30 amplification cycles (95 ^{o}C for 20 s, 56 ^{o}C for 30 s, 72 ^{o}C for 60 s) and final elongation step at 72 ^{o}C for 5 min. Duplicate PCR reactions were performed for each sample, with the duplicates pooled after PCR. The adaptors contain 16S V1-3 specific primers: [27F] AGAGTTTGATCCTGGCTCAG and [534R] ATTACCGCGGCTGCTGG (3). The resulting amplicon libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter) with a bead to sample ratio of 4:5. DNA was eluted in 25 µL of nuclease-free water (Qiagen). Gel electrophoresis used Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent Technologies) to validate the product size and purity of a subset of sequencing libraries.

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 6 nM. The samples were paired-end sequenced (2x300 bp) on a MiSeq (Illumina, USA) using a MiSeq Reagent kit v3 (Illumina), following standard guidelines for preparing and loading samples on the MiSeq. >10% PhiX control library was spiked to overcome low complexity issues often observed with amplicon samples.

The forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (4) with the settings SLIDINGWINDOW:5:3 and MINLEN:275. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (5) with the settings -m 10 -M 200. The trimmed reads were dereplicated and formatted for use in the UPARSE workflow (6). The dereplicated reads were clustered, using the USEARCH v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the USEARCH v. 7.0.1090 -usearch_global command with -id 0.97 -maxaccepts 0 -maxrejects 0.

SI.4. Material and methods: Biofilm characterization and imaging

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to observe the surface structure and morphology of the biofilm that developed on the front and back of the WFMF membrane after 47 days of operation of the systems, as previously described (7,8). A filter sample of approximately 1 cm^2 was extracted and fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS), rinsed with PBS, post-fixed with 1% OsO_4 in PBS for 30 mins, rinsed again with PBS, and dehydrated using ethanol (70% in H_2O for 30 mins, 100% for 30 mins). Subsequently, ethanol was exchanged with hexamethyldisilazane and the sample was dried overnight. The piece was sputter-coated with 4 nm of platinum (CCU-010, Safematic, Switzerland) and analyzed in a field emission scanning electron microscope (Zeiss Supra 50 VP, Oberkochen, Germany) using the secondary electron detector.

Confocal Laser Scanning Microscopy

As previously described, Confocal Laser Scanning Microscopy (CLSM) was used to characterize the distribution of live and dead cells on the membrane (8). After 36 days of operation, a 1 cm^2 sample of the fouled membrane was extracted from WFMF reactor B and stained using a 1:2000 dilution of Hoechst 33342 nucleic acid gel stain (Thermo Fisher, Switzerland) in PBS to detect dead and live cells. The fluorescence of the Hoechst stain was detected by excitation at 351 nm and emission at 461 nm. The images were captured using a Leica SP5 microscope equipped with the 40x/0.8 water immersion Leica objective.

Optical Coherence Tomography (OCT)

Optical Coherence Tomography (OCT) was used to investigate the biofilm structure at the mesoscale level (mm) (7). Filtration modules were carefully removed from WFMF reactor B after 36 days of operation and immersed in filtered Evian water. The images were captured using the 930 nm Spectral Domain OCT (Thorlabs GmbH, Dachau, Germany) with a central light source wavelength of 930 nm. The OCT images were processed to remove background noise using ImageJ software.

SI.5. Results: Physicochemical Performance

Table S1. *Results from total organic carbon (TOC), total inorganic carbon (TIC), and chemical oxygen demand (COD) analyses in reactors A and B, after 10 days of system start-up and until the last week of operation.*

	TOC (mg/L), n=5	TIC (mg/L), n=5	COD (mg/L), n=4
Influent A	7.45 (0.91)	82.24 (2.78)	29.45 (11.96)
Permeate A	6.98 (2.12)	81.11 (4.82)	21.60 (9.31)
% Removal	7.7% (14.7%)	1.4% (4.1%)	20.1% (28.8)
Influent B	6.89 (0.28)	82.89 (3.94)	28.53 (14.65)
Permeate B	6.81 (2.15)	84.60 (5.52)	24.75 (9.48)
% Removal	1.2% (26.4%)	-2.0 % (2.2%)	5.2% (35.2%)
Mean A & B % removal	4.5%	0.3%	12.6%

Table S2. *Results from the total nitrogen (TN), nitrate (NO_3^-), phosphate (PO_4^{3-}), sulfate (SO_4^{2-}), and total phosphorus (TP) analyses in reactors A and B, after 10 days of system start-up and until the last week of operation. The concentrations of ammonium (NH_4^+) and nitrite (NO_2^-) were consistently below their detection limits of 0.2 and 0.5 mg/L, respectively, and are not shown in the table. Values in () are standard deviations.*

	TN (mg/L as N), n=5	NO_3^- (mg/L of N), n=5	TP (mg/L of P), n=4	PO_4^{3-} (mg/L of P) n=5	SO_4^{2-} (mg/L), n=5
Influent A	5.95 (0.92)	4.22 (0.87)	3.07 (1.29)	1.92 (0.16)	39.66 (2.13)
Permeate A	3.23 (1.03)	2.12 (1.11)	2.46 (0.44)	1.76 (0.27)	39.04 (3.76)
% Removal	43.8% (20.9%)	50.1% (24.8%)	15.1% (14.0%)	8.7% (8.1%)	1.7% (5.2%)
Influent B	3.77 (2.11)	2.58 (1.69)	2.39 (0.51)	1.58 (0.11)	39.68 (2.90)
Permeate B	1.38 (0.74)	0.66 (0.54)	2.23 (0.47)	1.44 (0.25)	39.64 (2.62)
% Removal	57.3%	73.1% (8.0%)	5.0% (17.2%)	9.2% (10.2%)	0.0% (1.7%)
Mean A & B % removal	49.8%	61.6%	10.1%	8.9%	0.87%

***Table S3**. Results from the chlorine (Cl^-), sodium (Na^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) analyses in reactors A and B, after 10 days of system start-up and until the last week of operation. Values in () are standard deviations.*

	Cl^- (mg/L), n=5	**Na^+ (mg/L), n=5**	**K^+ (mg/L), n=5**	**Ca^{2+} (mg/L), n=5**	**Mg^{2+} (mg/L), n=5**
Influent A	238.80 (12.40)	183.40 (4.04)	12.96 (0.36)	108.80 (3.90)	22.44 (0.60)
Permeate A	229.20 (20.54)	174.60 (3.78)	12.94 (1.19)	103.00 (3.24)	21.30 (0.80)
% Removal	4.0% (6.6%)	4.8% (2.9%)	0.0% (9.51%)	5.3% (2.2%)	5.1% (3.1%)
Influent B	229.40 (28.09)	173.60 (7.13)	13.02 (1.50)	103.10 (5.05)	20.94 (0.84)
Permeate B	227.40 (27.83)	175.40 (8.11)	13.02 (1.50)	104.80 (7.05)	21.52 (1.15)
%Removal	0.8% (2.0%)	-1.1% (3.2%)	1.8% (18.2%)	-1.7% (6.1%)	-2.8% (5.0%)
Mean A & B % removal	2.4%	1.8%	0.9%	1.8%	1.1%

***Table S4**. pH, and temperature changes in reactors A and B. Values in () are standard deviations.*

	pH, n=4	**Temp. (C°), n=5**
Influent A	8.81 (0.10)	24.96 (1.77)
Permeate A	8.48 (0.22)	26.93 (1.83)
Influent B	8.91 (0.06)	24.90 (1.76)
Permeate B	8.59 (0.19)	26.98 (1.51)

SI.6. Results: Cell counts and ATP assay

***Table S5**. Flow cytometry measurements of intact cell count (ICC) and total cell count (TCC) at the influent and permeate of reactors A and B in units of cells/mL.*

	Influent A		Influent B		Permeate A		Permeate B	
Day	ICC	TCC	ICC	TCC	ICC	TCC	ICC	TCC
2	1.00E+06	1.79E+06	3.33E+05	5.97E+06	1.36E+06	3.65E+06	6.53E+05	1.30E+06
9	4.63E+05	3.53E+06	5.00E+05	1.67E+06	5.00E+05	2.32E+06	4.93E+05	2.70E+06
16	1.30E+06	2.47E+06	1.43E+06	1.43E+06	8.27E+05	3.76E+06	7.63E+05	2.07E+06
20	1.91E+06	3.07E+06	8.63E+05	1.71E+06	9.30E+05	4.25E+06	5.00E+05	1.81E+06
37	1.78E+06	4.95E+06	2.78E+06	7.49E+06	1.12E+06	1.51E+06	6.02E+05	2.53E+06

Table S6. *Intracellular ATP (ATPI) and total ATP (ATPT) at the influent and permeate of reactors A and B, in units of nM.*

	Influent A		Influent B		Permeate A		Permeate B	
Day	ATP_I	ATP_T	ATP_I	ATP_T	ATP_I	ATP_T	ATP_I	ATP_T
5	-	-	-	-	4.00	5.78	2.96	5.43
6	3.45	7.51	1.35	2.78	10.70	13.80	1.97	6.32
14	3.49	7.15	3.66	7.61	3.38	7.69	2.12	7.99
16	2.93	4.80	1.92	2.29	0.62	1.30	0.30	1.70
19	5.06	5.82	3.12	4.33	1.41	1.69	0.30	1.96
21	3.57	3.65	2.63	2.74	1.17	1.66	0.81	1.13
25	6.02	6.60	4.55	5.08	1.14	1.39	2.03	2.28
30	4.32	4.99	0.63	1.35	0.84	1.39	0.84	1.07
38	4.45	4.94	2.02	2.54	1.47	1.82	0.82	1.16
40	5.59	6.51	4.42	5.26	2.48	2.65	1.25	1.42

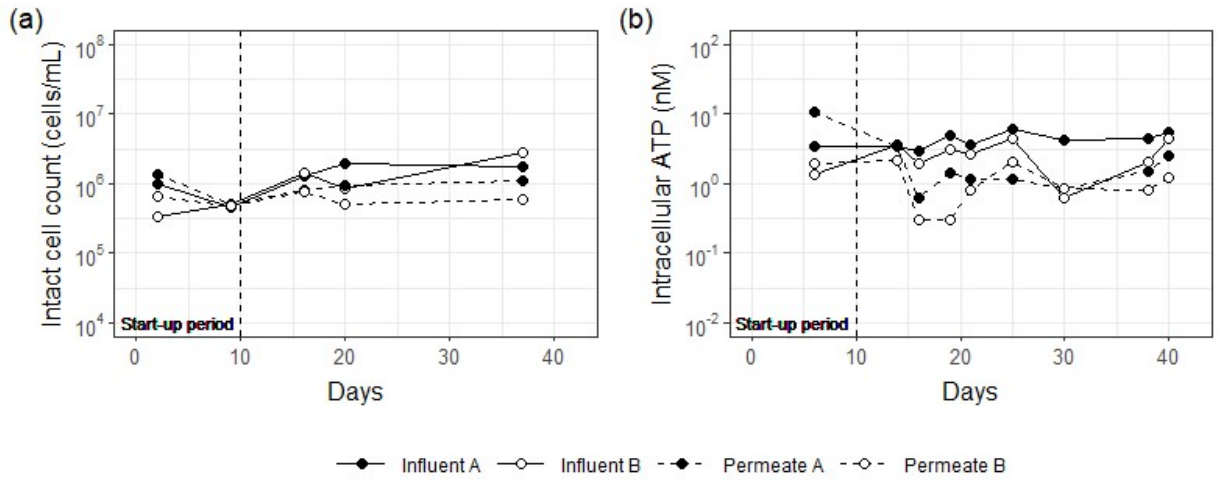

Figure S1*. Time series of (a) intact cell count and (b) intracellular ATP at the influent and permeate of reactors A and B.*

SI.7. Results: Relative abundances by phylum, class, and genus

Table S7. *Relative abundances by phylum. Only phyla with relative abundance higher than 1% in at least one sample are included. The table shows the arithmetic means by sampling location.*

Phylum	Influent	Biofilm	Permeate
Pseudomonadota	78.8%	48.9%	47.2%
Bacillota	8.9%	4.7%	0.6%
Bacteroidota	7.1%	7.0%	2.7%
Saccharibacteria	1.0%	0.3%	1.0%
TM6	1.0%	0.5%	0.4%
Actinomycetota	0.7%	11.1%	13.4%
Chloroflexota	0.4%	13.1%	19.5%
Planctomycetota	0.2%	5.7%	4.3%
Cyanobacteria	0.0%	1.2%	0.8%
Acidobacteriota	0.0%	1.6%	5.7%
Nitrospirota	0.0%	3.1%	0.6%
Gemmatimonadota	0.0%	1.0%	1.7%
Other/non-identified phyla	1.8%	1.9%	2.1%

Table S8. *Relative abundances by class. Only classes with relative abundance higher than 1% in at least one sample are included. The table shows the arithmetic means by sampling location.*

Class	Influent	Biofilm	Permeate
Betaproteobacteria	43.8%	8.4%	14.9%
Alphaproteobacteria	21.9%	28.0%	24.9%
Gammaproteobacteria	12.5%	8.9%	4.6%
Clostridia	7.8%	4.0%	0.5%
Flavobacteriia	6.6%	0.3%	0.7%
Actinobacteria	0.6%	9.9%	12.6%
Deltaproteobacteria	0.4%	3.1%	2.5%
Sphingobacteriia	0.4%	2.2%	1.0%
Anaerolineae	0.1%	0.4%	1.5%
Caldilineae	0.1%	6.6%	14.0%
Thermomicrobia	0.1%	4.8%	2.0%
Planctomycetacia	0.1%	3.5%	2.0%

Acidobacteria	0.0%	1.6%	5.5%
Cytophagia	0.0%	4.4%	1.1%
Nitrospira	0.0%	3.1%	0.6%
Gemmatimonadetes	0.0%	1.0%	1.7%
Phycisphaerae	0.0%	1.8%	1.9%
Chloroplast	0.0%	0.9%	0.6%
JG30-KF-CM66	0.0%	0.1%	0.6%
Other/non-identified classes	5.7%	6.9%	6.9%

Table S9. *Relative abundances by genus. Only genera with relative abundance higher than 1% in at least one sample are included. The table shows the arithmetic means by sampling location.*

Genus	Influent	Biofilm	Permeate
Novosphingobium	14.4%	0.1%	0.7%
Legionella	7.7%	0.6%	0.6%
Flavobacterium	6.5%	0.1%	0.6%
Romboutsia	4.8%	2.4%	0.3%
Polynucleobacter	4.1%	0.0%	0.3%
Limnohabitans	2.9%	0.0%	0.2%
Sphaerotilus	2.7%	0.8%	0.7%
MNG7	2.4%	1.8%	1.2%
Clostridium sensu stricto 1	2.1%	1.1%	0.1%
Acidovorax	0.2%	0.4%	0.5%
Blastomonas	0.2%	0.3%	0.5%
Hydrogenophaga	0.2%	1.0%	2.6%
Reyranella	0.1%	2.1%	0.8%
Rhodococcus	0.1%	3.9%	5.2%
Hyphomicrobium	0.1%	4.3%	2.5%
Planctomyces	0.0%	1.3%	0.6%
Marinicella	0.0%	3.0%	0.2%
Mycobacterium	0.0%	1.3%	3.0%

Sphingobium	0.0%	0.1%	0.5%
Variibacter	0.0%	1.5%	1.3%
Gordonia	0.0%	1.2%	0.6%
Aquincola	0.0%	0.0%	1.2%
Nitrospira	0.0%	3.1%	0.6%
C1711WL	0.0%	0.2%	0.9%
Xylophilus	0.0%	0.0%	1.7%
Pedomicrobium	0.0%	1.5%	1.5%
Arenimonas	0.0%	1.3%	0.1%
Nitratireductor	0.0%	1.0%	0.2%
SM1A02	0.0%	0.85%	1.0%
Nocardia	0.0%	0.61%	1.9%
Steroidobacter	0.0%	0.27%	0.7%
Blastocatella	0.0%	0.21%	0.7%
Lysinimonas	0.0%	0.01%	0.6%
Other/non-identified genera	51.5%	63.7%	66.1%

SI.8. Results: Biofilm characterization and imaging

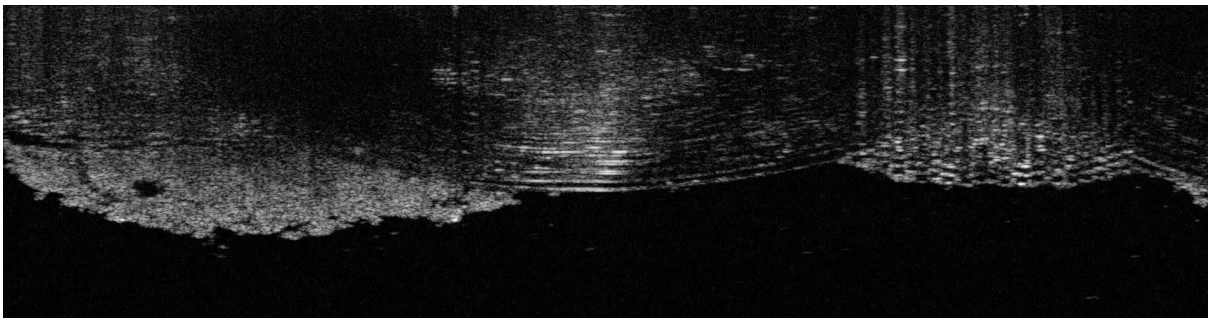

Figure S2. *Cross-sectional image of WFMF membrane taken with Optical Coherence Tomography after 36 days of continuous operation.*

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