Electronic Supplementary Information
Bacterial transmission and colonization in activated carbon block (ACB) point-of-use (PoU) filters

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This supporting information contains a 24-page document, including operational program, analytical methods, 8 figures, 4 tables, references and this cover page.

Contents

Operational Program of the filter manifold system

Bacteria experiment

Every filter drew 0.5 L of influent each hour for 11 hr, followed by a 13-hr stagnation period. The bacteria experiment was conducted for a total of 29 days. The first 23 days was an application phase during which fluorescent bacteria were mixed with the tap water. The last 6 days was a washout phase when no supplemental fluorescent bacteria were added. The operation of application phase during each hour is as follows:

1. 2 L tap water was flushed through the pipes to the waste. The purpose of this step is to flush out the stagnant water from the filter manifold system.
2. 2.3 L of tap water was pumped into the influent reservoir.
3. The bacterial stock suspensions were stored in two 60 mL syringes. 2 mL of fluorescent bacteria suspension was injected to the influent reservoir by syringe pump (Chemyx, Stafford, TX).
4. Tap water was mixed with the fluorescent bacteria suspension in the influent reservoir for 2 min.
5. Around 500 mL influent was pumped to each filter. Each pumping time lasted 5 to 7 sec.
6. The rest of the influent in reservoir was drained as waste.

Microsphere experiment

Every filter drew 0.5 L of influent each hour for 16 hr, followed by an 8-hr stagnation period. The microsphere experiment was conducted for 13 days. The operation during each hour was similar to the fluorescent bacteria experiment except two modifications:

1. Deionized water was used to flush through the pipes and feed to the manifold system.
2. The volume of microsphere suspension for dilution was 3 mL.

Analytical methods

Flow cytometry

The flow cytometer used for fluorescent-tagged bacteria and fluorescent microspheres was a ZE5 cell analyzer (Bio-Rad, CA). The instrument was calibrated before each use. The instrument was set to agitate for 5 s per sample to maintain cell suspension. All samples were injected into the flow cytometer with a flow rate of 1 μL/s. The analysis of populations was carried out using the Everest Flow cytometry analysis software. Well-dispersed suspended *E. coli* and *P. aeruginosa* solutions were used to determine gate parameters of individual cell counts. To gate the fluorescent bacteria, the forward scatter of the two fluorescent bacteria was detected by the 405 nm forward scatter small particle detector set at a photomultiplier tube voltage of 347 V with linear gain. Green fluorescence emitted from *P. aeruginosa* was detected via the GFP detector set at the voltage of 491 V with logarithmic gain. The red fluorescence emitted from *E. coli* was detected via the mCherry detector of 587 V with logarithmic gain. The acquisition of each sample count stopped when a total of 1,000 events was acquired or the flow-through volume reached 300 μL. The detection limit of the two fluorescent bacteria was tested by serially diluting the acclimated fluorescent bacterial suspensions with a buffer solution containing 3 mM potassium dihydrogen (Fisher Scientific, Pittsburgh, PA) and 4 mM magnesium chloride (Fisher Scientific, Pittsburgh, PA). Triplicate samples were tested, and the coefficient of variance was lesser than 10%. The R^2 of the standard curve is 0.99. The lowest diluted suspension that can be detected was 50 cell/mL and was taken to be the detection limit of the instrument. The flow cytometry dot plots of detection limit and examples of the influent and effluent samples are shown in Figure S1-S2.

Microsphere fluorescence was detected via a GFP detector with a voltage of 391 V. The forward and side scatter of the microspheres were collected by the 488 nm forward scatter detector and 488

nm side scatter detector in linear gain with the voltage of 365 V and 675 V. The acquisition of each sample stopped when 10,000 events or a 40 µL flow-through volume was reached. Example flow cytometry dot plots of the influent and effluent samples are given in Figure S3. Flow cytometry dot plots of the influent and effluent samples also confirmed that the microspheres did not noticeably aggregate (Figure S3), since we did not observe a separate population showing at the upper right hand corner of the plot.

Total bacterial measurements were conducted using a BD Accuri C6 Plus flow cytometer (BD Accuri cytometers, Belgium). The instrument was calibrated before each use. Bacterial cells were stained by adding 1 µL of SYTO Green (1,000X) and 20 µL of propidium iodide (PI, 1 mg/mL, 1.5 mM) into 979 µL of water sample to obtain 1X SYTO Green and 0.03 mM PI in the final solution. SYTO® Green stains all bacterial cells irrespective of membrane damage. PI stains only the membrane-compromised cells. After the addition of the dyes, samples were vortexed and incubated in a dark environment at room temperature for 15 min. A total of 40 µL of each sample was measured at a flow rate of 14 µL/min using fluorescent detectors 1 (FL1: 533 ± 30 nm) and 3 (FL3: > 670). SYTO® Green allows discrimination between fluorescent cells and non-fluorescent debris in the FL1 detector. PI was detected in the FL3 detector (emission filter > 670 nm). A threshold value of 800 was applied on the FL1 channel. All data were analyzed using BD Accuri C6 software.

Culturing viable fluorescent-tagged bacteria

To evaluate the presence of culturable bacteria, 50 mL water sample volumes were serially diluted to 100X and then filtered through a 0.45 µm mixed cellular ester membrane filter (Millipore, Billerica, MA). The membrane filters were placed onto Pseudomonas isolation agar (Fisher Scientific, Hampton, NH) or M Endo LES agar (Fisher Scientific, Hampton, NH) [1] and incubated

at 35^0 C for 24 and 48 hours, respectively [1,2]. The presence of *P. aeruginosa* was indicated by green colonies; coliform colonies presented with a pink to dark-red color and metallic surface sheen. To validate the presence of *E. coli*, the dark-red and gold colonies cultured from M Endo LES agar were transferred to nutrient agar with 4-methylumbelliferyl-β-D-glucuronide (MUG). *E. coli* colonies produce the enzyme β-glucuronidase and hydrolyze the MUG substrate to produce a blue fluorescence around the periphery of the colony under long-wave UV light at 365 nm.

Quantitative PCR (qPCR)

The extracted DNA from the effluent water samples and biofilm from the filter inner channel walls and ACB fabric mesh was analyzed by qPCR targeting GFP and mCherry genes. All analyses were performed in triplicate. A standard curve was generated by amplifying 7-fold serially diluted GFP-tagged *P. aeruginosa* or mCherry-tagged *E. coli* with a known amount of gene copies for each targeted gene. All qPCR experiments were carried out on an Eppendorf® Mastercycler (Eppendorf, Germany). The PCR mixture (10 μL) consisted of Fast-Plus EvaGreen Master Mix (Biotium, CA, US), primer sets (0.5 μM each, Table 5-1), 0.625 mg/mL BSA, and 1 μL template DNA. The reaction conditions were estimated based on primer sequences and features of the polymerase used. The following conditions were ultimately used: 95°C for 2 min, followed by 35 cycles at 95°C for 5 sec, annealing for 5 sec at a defined temperature (shown in Table 1) and 72°C for 25 sec. The qPCR efficiency of the two genes was 90-94%, and R^2 values were 0.99 for the standard curves. A melting curve was used to confirm the specificity of qPCR-amplified fragments.

Measurements of bacterial cell size

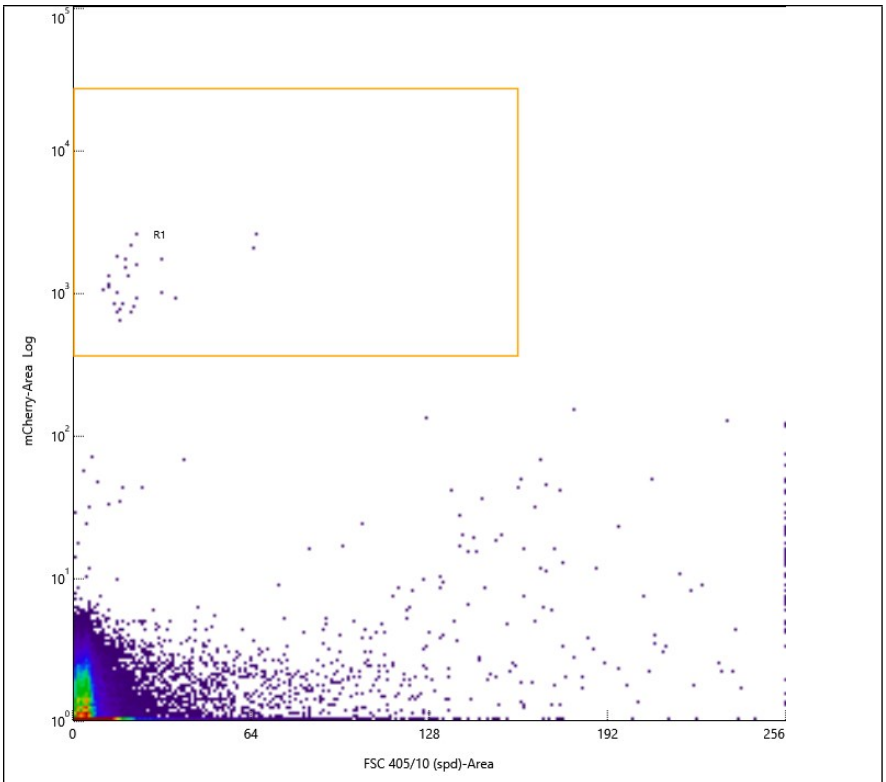
In order to analyze the size of fluorescent bacteria before and after acclimation, phase-contrast images were taken using a microscope (Axio observer Z1, Zeiss, Oberkochen, Germany). The GFP signal of *P. aeruginosa* was measured using an excitation filter at 470 nm and emission at 525 nm. The mCherry signal of *E. coli* was measured using an excitation filter at 546 nm and emission at 590 nm. For photographs, images were obtained using a 100× oil immersion objective and a numerical aperture of 1.3. The images were processed using ImageJ software to determine the length and width of a minimum of 100 cells.

Bacterial adhesion to hydrocarbons (BATH) assay

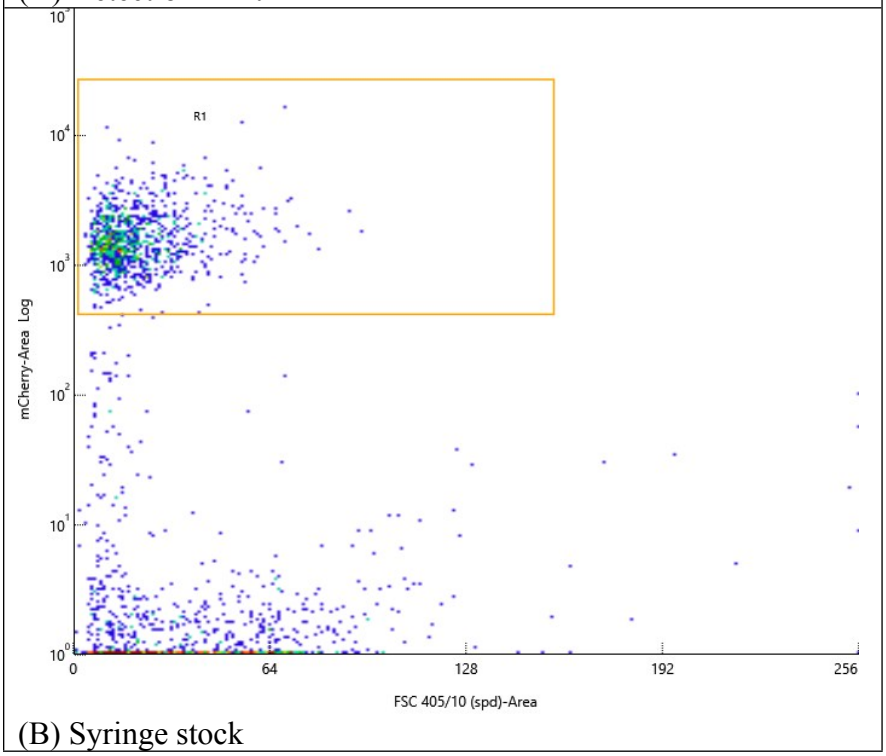
BATH assays were performed as described by [3] with modification. The fluorescent bacteria before and after acclimation were tested using iso-octane as the hydrocarbon. Three milliliters of the bacterial cell suspensions was added to 1 mL of HPLC-grade iso-octane (Sigma-Aldrich, S. Louis, MO), followed by addition to 1 mL of 3 M ammonium sulfate $(NH_4)_2SO_4$) (Fisher Scientific, Atlanta, GA). The mean initial concentration of the bacterial cell suspension before and after acclimation was 7.6×10^7 and 6.8×10^5 cell/mL, respectively. Suspensions were vortexed for 3 min. A centrifuge tube containing 3 mL of untreated cell suspension served as a control (*Ac*). All tubes were allowed to stand at room temperature for 30 min. After incubation for 30 min, 1 mL of the lower aqueous layer was removed using a pipette and the cell concentration (*Ab*) of each sample was measured by the aforementioned flow cytometry method. The ratio of the concentration of bacterial assay tubes to the control suspension was calculated as a percentage of cells bound to the hydrocarbon using the following equation:

$$\text{Percent cells adhering to hydrocarbon (\%)} = (Ac - Ab)/Ac \times 100 \quad (1)$$

Supplemental Figures

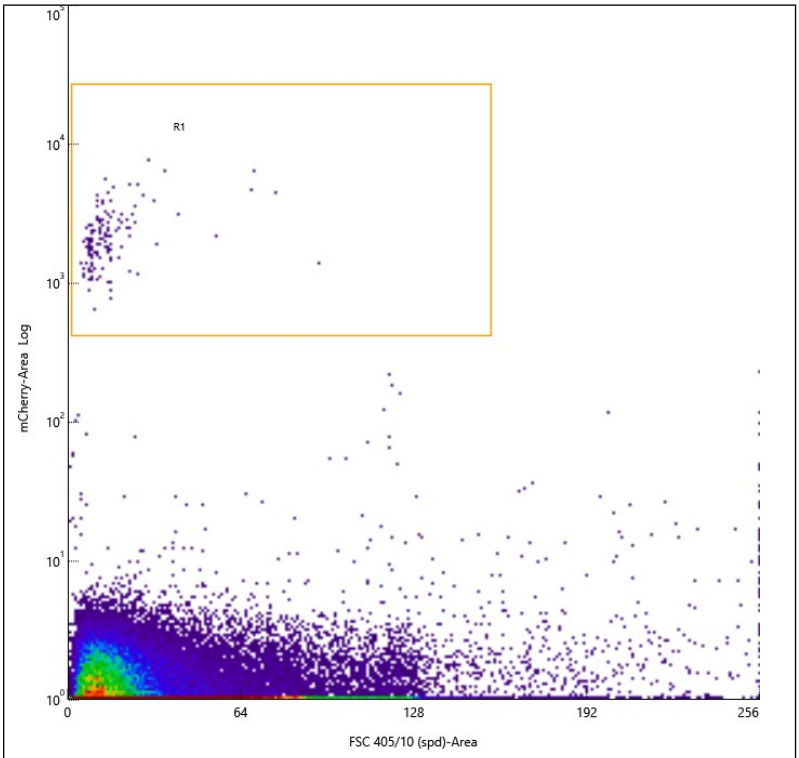


(A) Detection limit

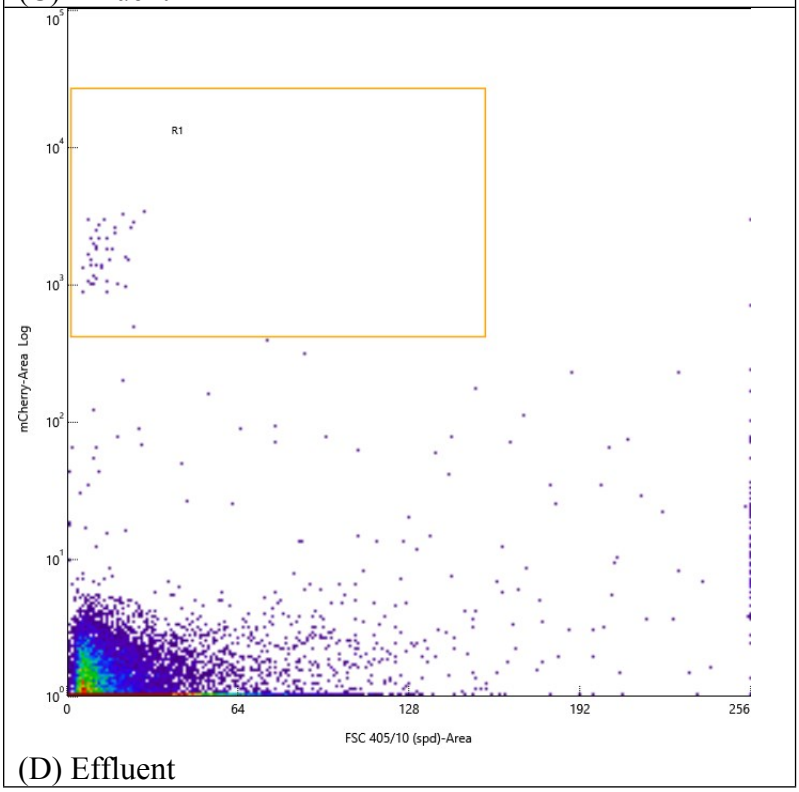


(B) Syringe stock

Figure S1 Flow cytometry dot plots of mCherry-tagged E. coli.

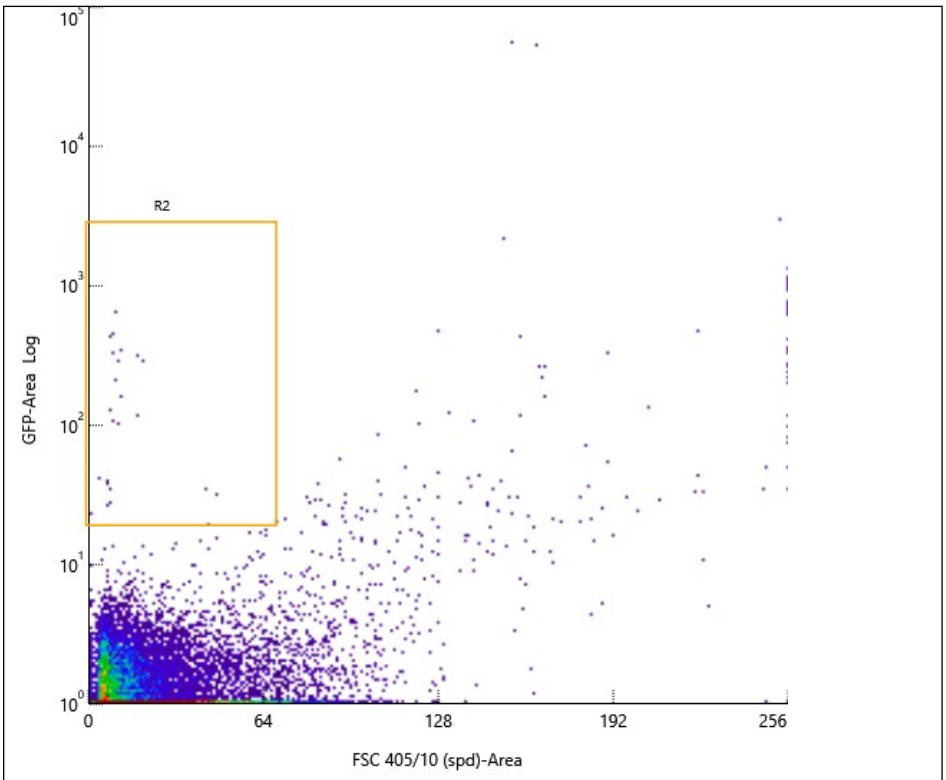


(C) Influent

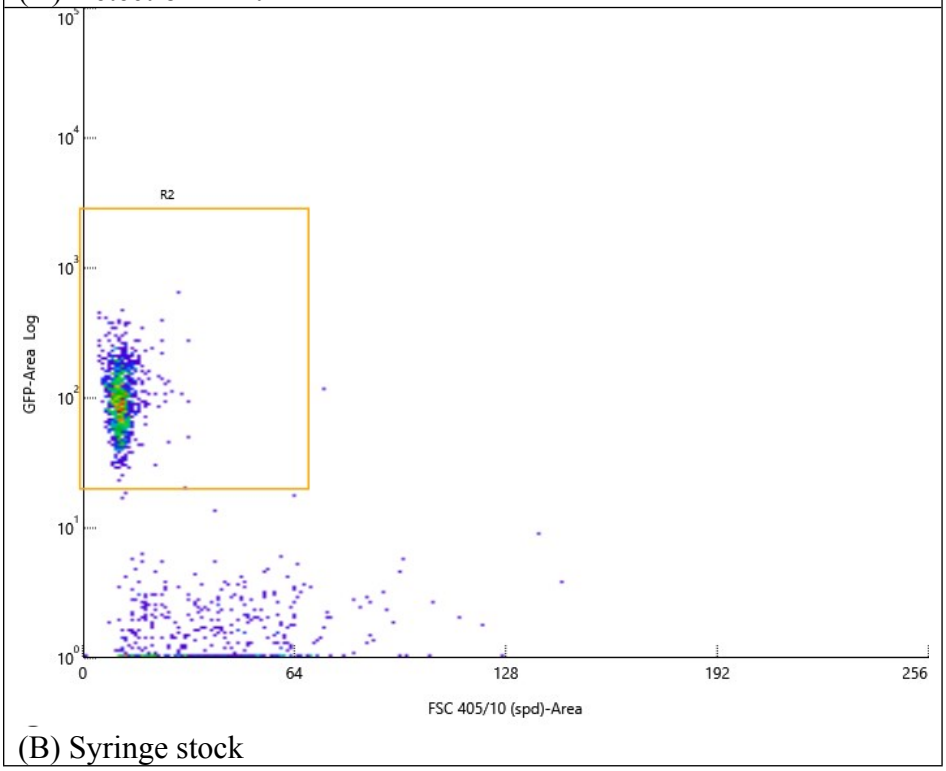


(D) Effluent

Figure S1 **Flow cytometry dot plots of mCherry-tagged *E. coli* (continuous)**

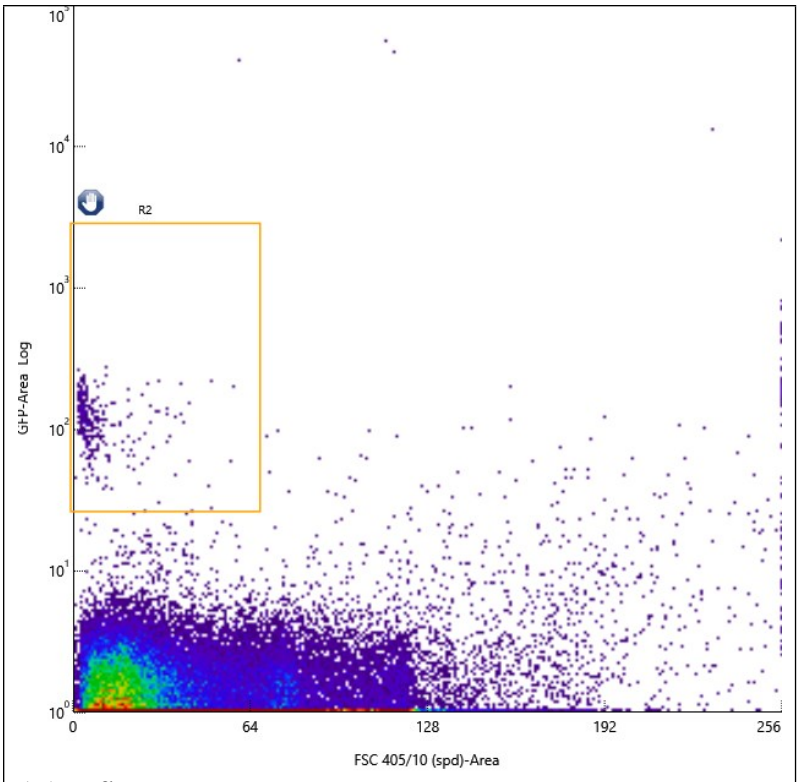


(A) Detection limit

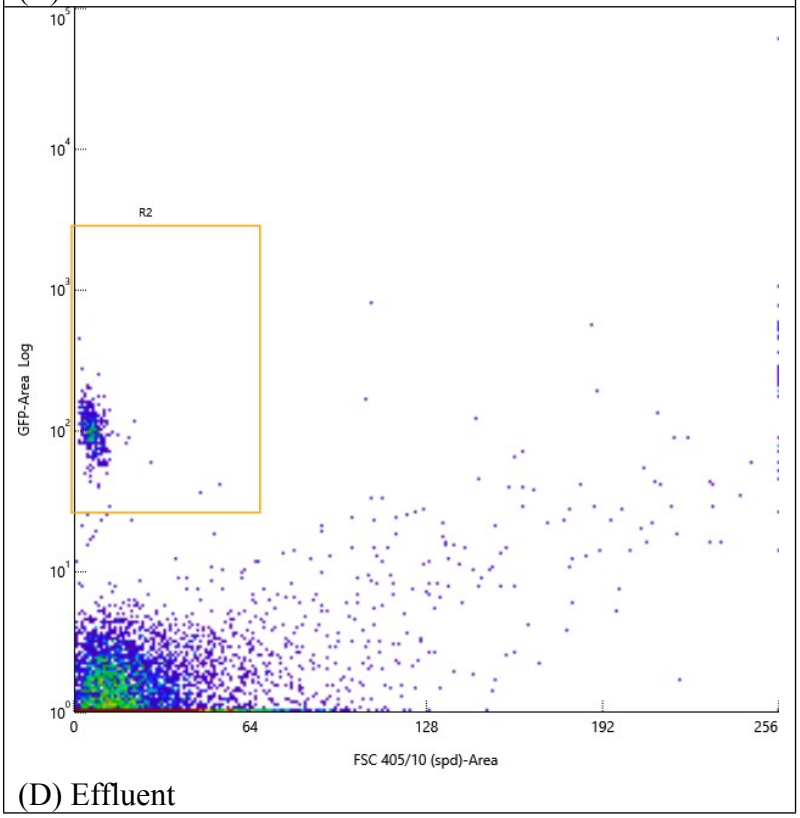


(B) Syringe stock

Figure S2 **Flow cytometry dot plots of GFP-tagged *P. aeruginosa*.**

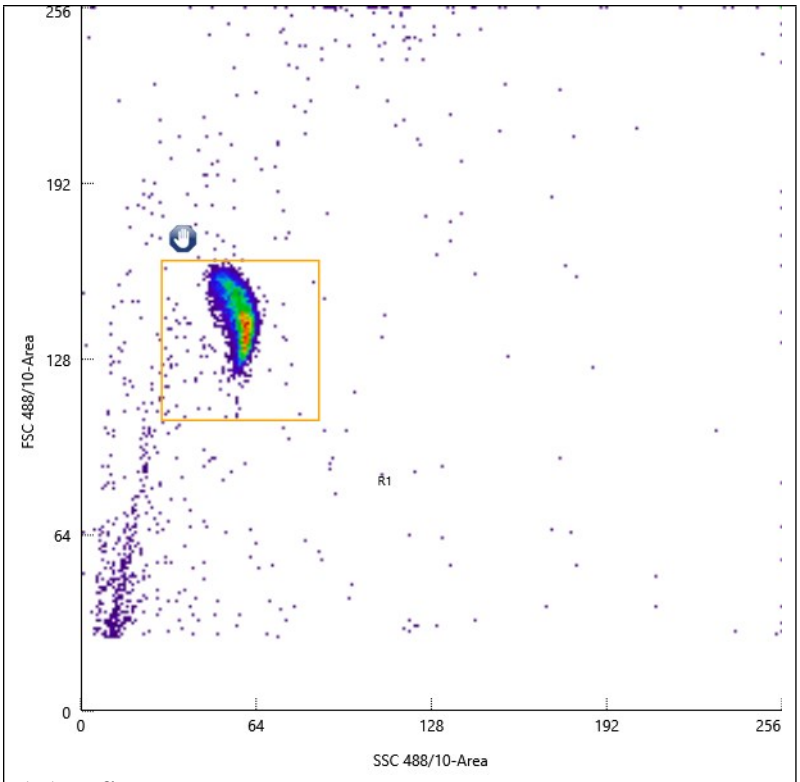


(C) Influent

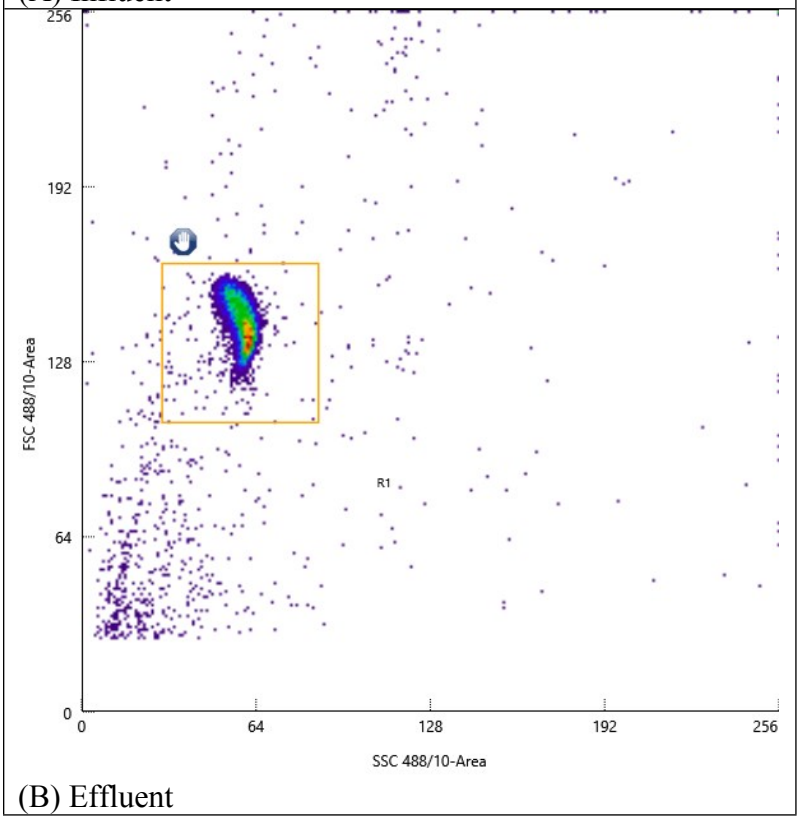


(D) Effluent

Figure S2 **Flow cytometry dot plots of GFP-tagged *P. aeruginosa* (continuous)**

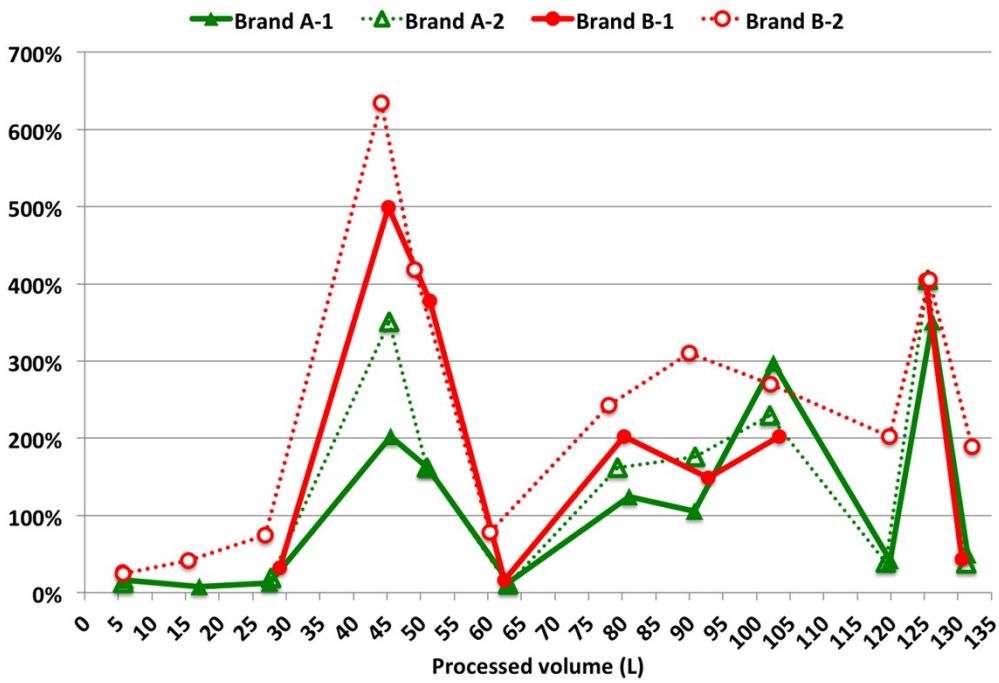


(A) Influent

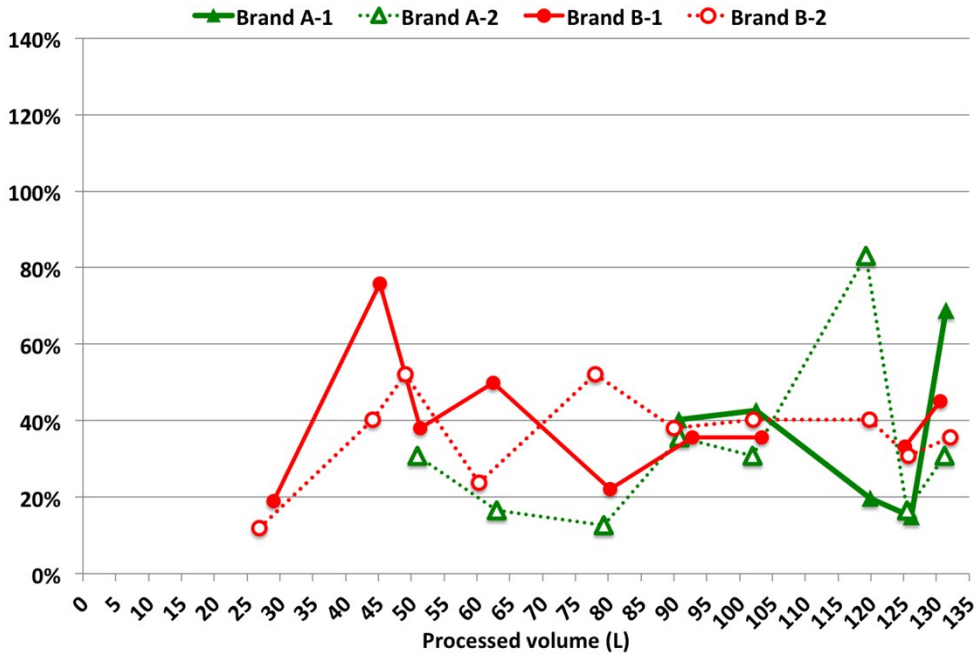


(B) Effluent

Figure S3 Flow cytometry dot plots of fluorescent microspheres.

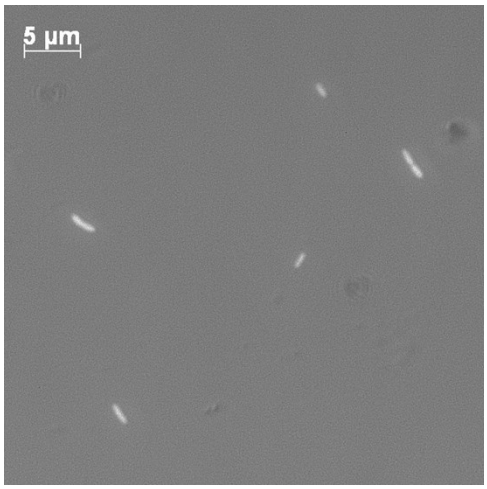


(A)

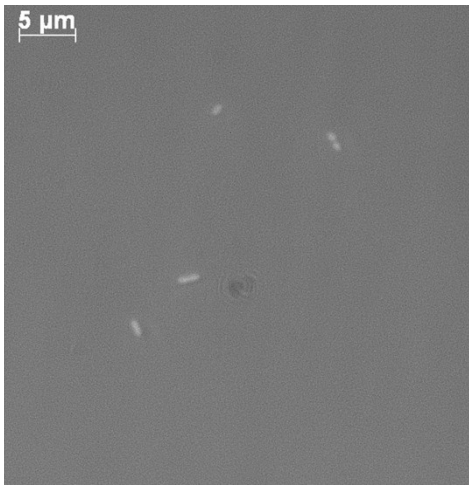


(B)

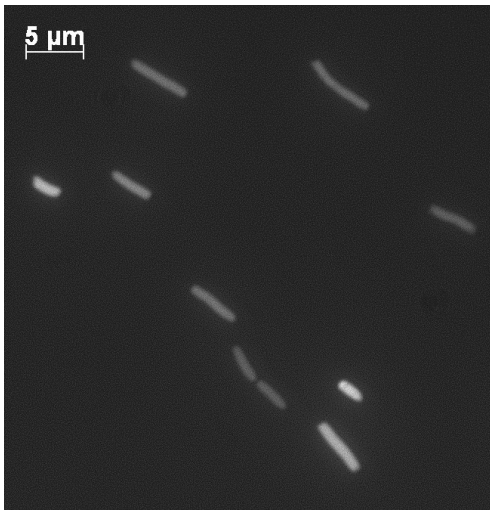
Figure S4 Ratio of effluent and influent concentration of (A) GFP-tagged P. aeruginosa and (B) mCherry-tagged E. coli through ACB PoU filters of Brand A (red) and Brand B (green).



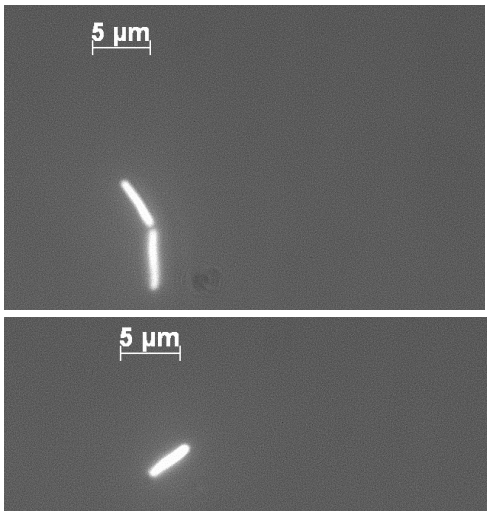
(A) *P. aeruginosa* before acclimation



(B) *P. aeruginosa* after acclimation



(C) *E. coli* before acclimation



(D) *E. coli* after acclimation

Figure S5 Microscopic visualization of P. aeruginosa and E. coli before and after acclimation to oligotrophic conditions.

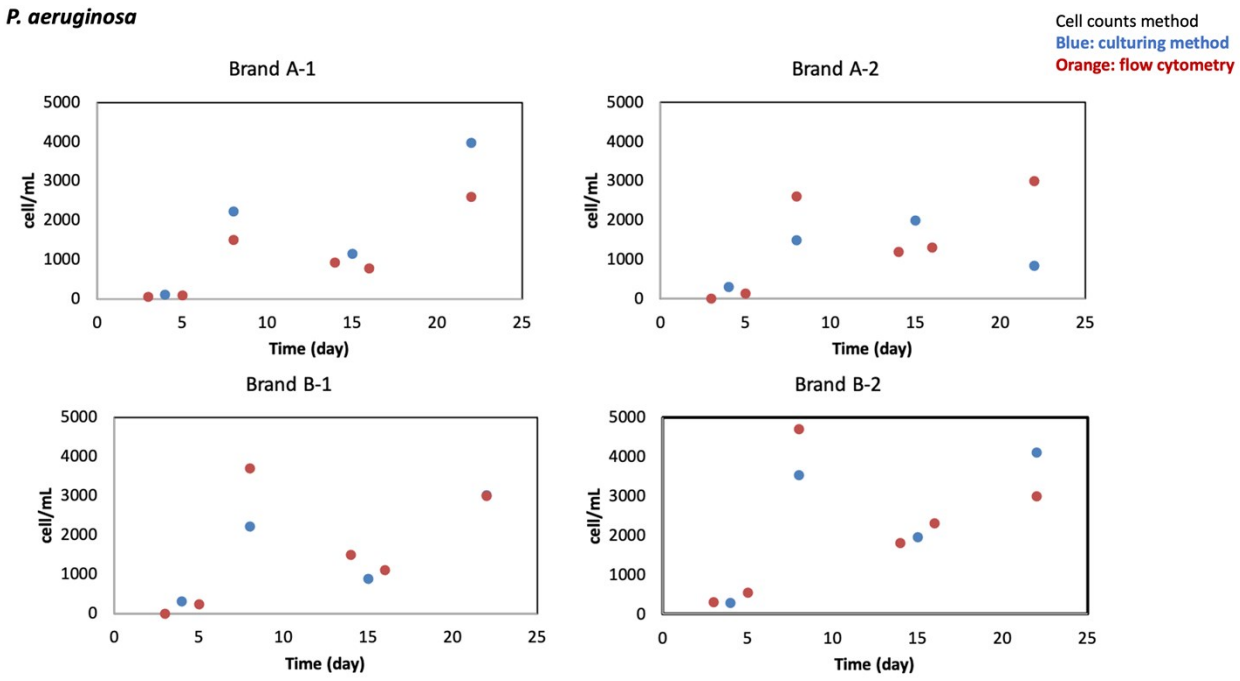


Figure S6 Culturability and flow cytometry cell counts of effluent P. aeruginosa.

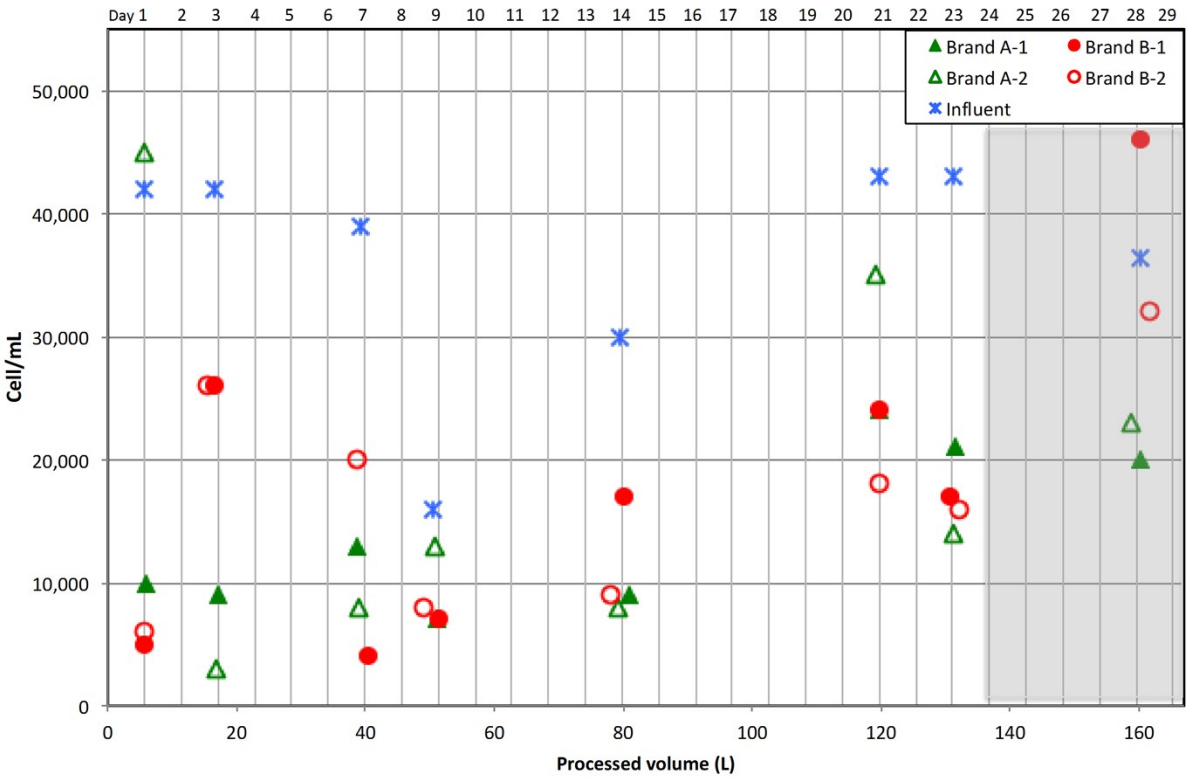
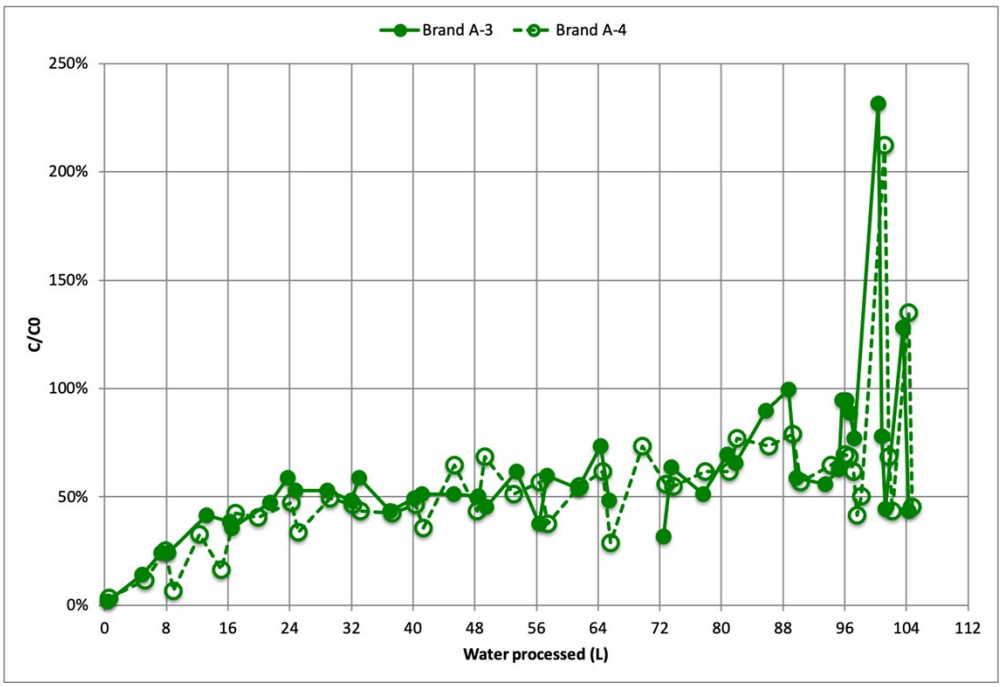
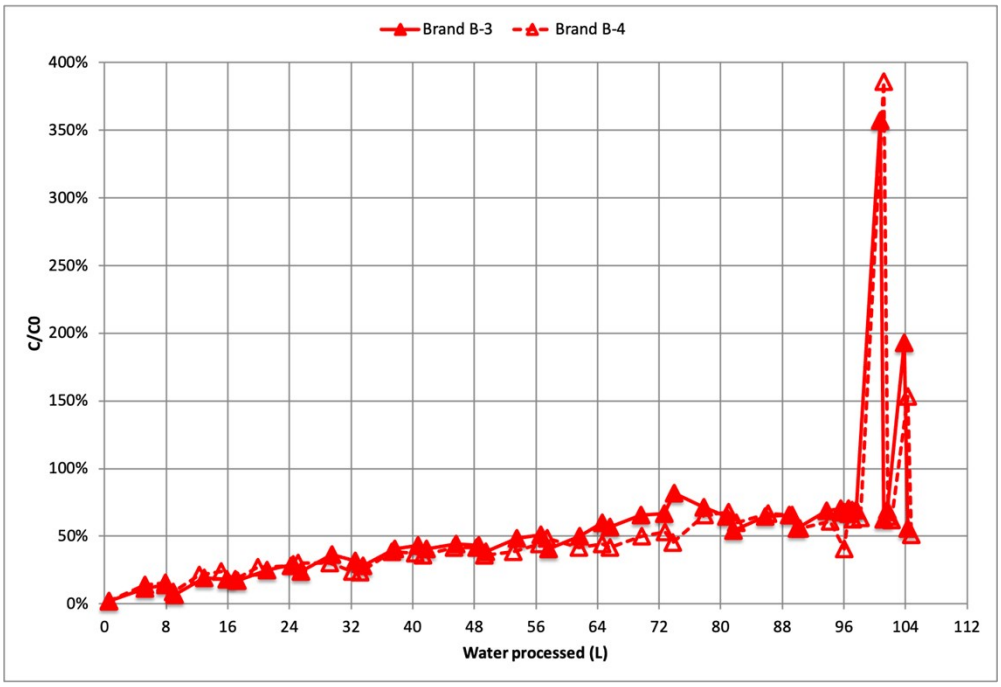


Figure S7 Breakthrough of membrane-intact bacteria (indigenous and fluorescent-tagged) through ACB PoU filters of Brand A (green) and Brand B (red). Inoculation of fluorescent-tagged bacteria stopped after day 23 (after process volume of 131 L).



(A)



(B)

Figure S8 Microsphere breakthrough pattern in duplicates of (A) Brand A and (B) Brand B filters. *When the filters of both brands had processed 96 L of the microsphere suspension influent, the filters began to clog and we observed that the effluent concentrations of microspheres of both brands reached two to four times the influent level. Given that all four of the filters were impacted simultaneously by clogging, the data for process volumes greater than 100 L was not considered in the empirical model.*

Supplemental Tables

Table S1 qPCR primers for two fluorescent chromosomal genes used in this study

Gene		Primer (5'-3')	Fragment size (bp)	Annealing Temperature (°C)	Reference
GFP	Forward	TGCCATGTG TAATCCCAG CA	97	55	This study
	Reverse	CTGTCCACACAATCTGCCCT			
mCherry	Forward	GGCGAAGAAGACAACATGGC	231	54	[18]
	Reverse	CGGATGCTTAACGTACGCTTTCG			

Table S2 Hydrophobicity of the fluorescent-tagged bacteria (N=3)

Species	Hydrophobicity (%)
P. aeruginosa	28±2
Acclimated *P. aeruginosa*	52±6
E. coli	5±1
Acclimated *E. coli*	58±6

***Table S3 Ranked permeabilities of ACB PoU filters used in bacterial breakthrough* experiments**

Filter label	Volume of water filtered after the initial 5 min (L)	Flow rate (L/min)	Rank of permeability*
A-1	25	5.1	2
A-2	25	5.1	2
B-1	26	5.2	1
B-2	24	4.9	3

*Before use in the breakthrough experiments, DI water was processed through each ACB PoU filter for 5 min. The rank of permeability was estimated by comparing the volume of water filtered over the first 5 min for each ACB PoU filter. The filter with the greatest volume of water processed was considered the most permeable.

Table S4 Cell balance of fluorescent-tagged bacteria across the filters

Parameters	GFP-*P. aeruginosa*				mCherry-*E. coli*			
Brand-Replicate #	A-1	A-2	B-1	B-2	A-1	A-2	B-1	B-2
Influent loading [a] (No. of cell)	$9.7\times10^7 \pm 4.6\times10^5$				$5.5\times10^7 \pm 2.6\times10^5$			
Integrated cell counts in the effluent over the spiking phase (No. of cell)	1.1×10^8	1.3×10^8	1.7×10^8	2.3×10^8	6.8×10^6	$1.2\times10_7$	1.8×10^7	1.7×10^7
The ratio of total cells eluted to total cells loaded	1.1	1.3	1.8	2.4	0.12	0.21	0.32	0.31
Estimated cell counts retained on fabric biofilm [b] (No. of cell each fabric)	5.0×10^3	2.8×10^3	1.8×10^4	6.6×10^4	5.3×10^4	$7.3\times10_4$	3.1×10^5	1.8×10^5

[a] Determined from the average influent concentration (N = 9) multiplied by the total volume of processed water (N = 9) .
[b] Estimated from the gene counts shown in Table 1. The estimated values pertain to the fabrics that were harvested after the washout phase. The size of fabric of Brand A and Brand B was 107 and 94 cm^2, respectively.

References

(1) APHA; AWWA; WEF. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washinton DC., 2005.

(2) Brown, V. I.; Lowbury, E. J. L. Use of an Improved Cetrimide Agar Medium and Other Culture Methods for Pseudomonas Aeruginosa. *J. Clin. Pathol.* **1965**, *18* (6), 752–756. https://doi.org/10.1136/jcp.18.6.752.

(3) Choi, N.-Y.; Bae, Y.-M.; Lee, S.-Y. Cell Surface Properties and Biofilm Formation of Pathogenic Bacteria. *Food Sci. Biotechnol.* **2015**, *24* (6), 2257–2264. https://doi.org/10.1007/s10068-015-0301-y.

(4) Englaender, J. A.; Jones, J. A.; Cress, B. F.; Kuhlman, T. E.; Linhardt, R. J.; Koffas, M. A. G. Effect of Genomic Integration Location on Heterologous Protein Expression and Metabolic Engineering in E. Coli. *ACS Synth. Biol.* **2017**, *6* (4), 710–720. https://doi.org/10.1021/acssynbio.6b00350.