Integrated assessment of particle associated fecal indicators elimination in algal-bacterial granular photobioreactors

❖ Supplementary methods

- **Quantification of FIB and coliphages using standard methods**

The quantification of FIB (*Escherichia coli* and *Enterococcus spp.*) was conducted by membrane filtration based on EPA Methods 1103.1. and 1600, respectively (Adhikari, Chhetri et al. 2020). Modified membrane-thermotolerant E. coli agar (mTEC; Difco, BD) was used for *Escherichia coli* measurement. The agar plates were incubated for 2 hr at 35 ± 0.5°C to revive impaired cells, followed by incubation for 24 ± 2 hr at 44.5 ± 0.5°C. For *Enterococcus spp.*, the membrane filters were placed on membrane-Enterococcus Indoxyl-β-D-Glucoside agar (mEI, Difco, BD) and incubated at 37 ± 0.5°C for 24 ± 2 hr. The concentrations were calculated as an average colony forming unit from plates with 30−300 colonies. Coliphages were evaluated using a double agar plaque assay based on the protocols described in 9224B and 9224C of Standard Methods (APHA SMWW, 2017). Somatic coliphage Phi X174 and Male-specific (F+) coliphage (MS2) were used as a surrogate for enteric phages to quantify the virus removal in the photobioreactors. In brief, a standard method to quantify virus infectious, a single colony of host bacteria (*E. coli Famp* (ATCC#700891) for Male-MS2 and *E. coli CN13* (ATCC#700609) for Phi X174 were inoculated into 10 mL tryptic soy broth media and incubated for 6 hr at 37 ±0.5 °C to prepare the host bacteria solution for the test. The stock nalidixic acid was added to all growth media for E. coli CN-13 and stock streptomycin/ampicillin was added to all growth media for *E. coli Famp*. 100 µL of this host bacteria solution was mixed with 500 µL of

0.22-μm filtered diluted samples and added to a liquid soft agar tube at 60 °C before pouring onto a base agar plate followed by incubation at 37 ±0.5 °C for 12-18 hr. After the incubation, the concentrations of coliphages were determined by counting the number of transparent circles in the opaque bacterial lawn, representing the sites of viral infection. Except for the very dilute effluent samples, the concentration was calculated as an average plaque count from plates with 10−200 plaques.

- **ROS Detection using DCFH-DA Probe**

Generally, ROS formation was measured by using the cell permeable probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The DCFH-DA is hydrolyzed by cellular esterases to form the nonfluorescent 2′,7′ -dichlorodihydrofluorescein (DCFH) after penetrating biomass cells, and then DCFH is immediately transformed to highly fluorescent 2′,7′ -dichlorofluorescein (DCF) in the presence of ROS. Considering that biomass collected from reactors is thick and cannot be used directly to evaluate ROS, the culture of ROS-producing algal-bacterial consortia was washed three times with phosphate-buffered saline (PBS) and resuspended and diluted in PBS to get 10^8 CFU/mL for ROS analysis. The reaction was performed in 96-well microplates. 100 μl of DCFH-DA was added per well containing 100 μl of 10^8 CFU/mL of resuspend cells and incubated for 45 min at 37°C in the dark. Then, the cells were collected by centrifugation and washed using an ROS assay buffer. The labeled cells were seeded in 100 μl of PBS and measured for the ROS upon completion, observing the cells immediately using fluorescence microscopy (Fig.S3 & Fig.S4).

- **The wastewater composition**

The synthetic wastewater composition was as follows in (g/L): CH_3COONa (0.6), NH_4Cl (0.31), K_2HPO_4 (0.073), and $NaHCO_3$ (1.1). Additionally, 1 ml of trace element

stock solution was added to the media, which consisted of (g/L): EDTA–$C_{10}H_{16}N_2O_8$ (0.5), $FeSO_4.7H_2O$ (0.2), $ZnSO_4.7H_2O$ (0.01), $MnCl_2.4H_2O$ (0.003), H_3BO_3 (0.03), $CoCl_2$ (0.011), $CuCl_2.2H_2O$ (0.162), $NiCl_2.6H_2O$ (0.002), $NaMoO_4.2H_2O$ (0.003), and $MgSO_4.7H_2O$ (0.02) in (g/L). The COD of real wastewater was 545 ± 25 mg/L, total nitrogen (TN) of 82 ± 4mg/L, total phosphorus (TP) of 14 ± 1mg/L, and the pH was 8.0 ± 0.1.

- **DNA extraction and sequencing data processed.**

The samples were stored at -80°C and genomic DNA was extracted using the FastDNA™ SPIN Kit for Soil. The quantity and quality of the genomic DNA were evaluated using Nanodrop (Thermo Scientific, Massachusetts, USA). PCR amplification and Next-Generation Sequencing targeting the 16S rRNA gene V4 variable region were performed by MR DNA® (Shallowater, TX, USA) using the Illumina platform. The universal bacterial primers 515/F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806/R (5′-GGACTACHVGGGTWTCTAAT- 3′) with a barcode on the forward primer were used to generate a 300 bp amplicon [72]. Sequencing data were processed using the QIIME2 (v2022.8) pipeline, including demultiplexing, trimming, and denoising with DADA2 [73]. Operational taxonomic units (OTUs) were generated with a 97% similarity cutoff, following the prokaryotic species concept [23]. To avoid potential PCR or sequencing errors, OTUs present at ≤0.05% abundance across all samples were bioinformatically removed.

❖ Supplementary Tables

Table S1: Summary of the bacterial richness and diversity of microbial communities.

Samples	OTU count	Shannon	Simpson	Chao1	ACE
PSBR-L	43907	5.29	0.99	390	390
PSBR-H	38585	4.97	0.98	332	332
Influent	50241	5.24	0.98	530	530
Effluent-PSBR-L	41721	5.24	0.99	359	359
Effluent-PSBR-H	32847	5.25	0.99	418	418

Table S2: Correlation analysis of ROS production and pathogens indicators in mixed liquor samples from the reactors during wastewater treatment operations

	ROS Production	*E.Coli*	*Enterococcus spp.*	F-specific coliphage	Somatic coliphage
ROS Production	1				
E.Coli	0.20	1			
Enterococcus spp.	0.54	0.63	1		
F-specific coliphage	0.31	0.62	0.81	1	
Somatic coliphage	0.74	0.25	0.64	0.65	1

❖ Supplementary Figures

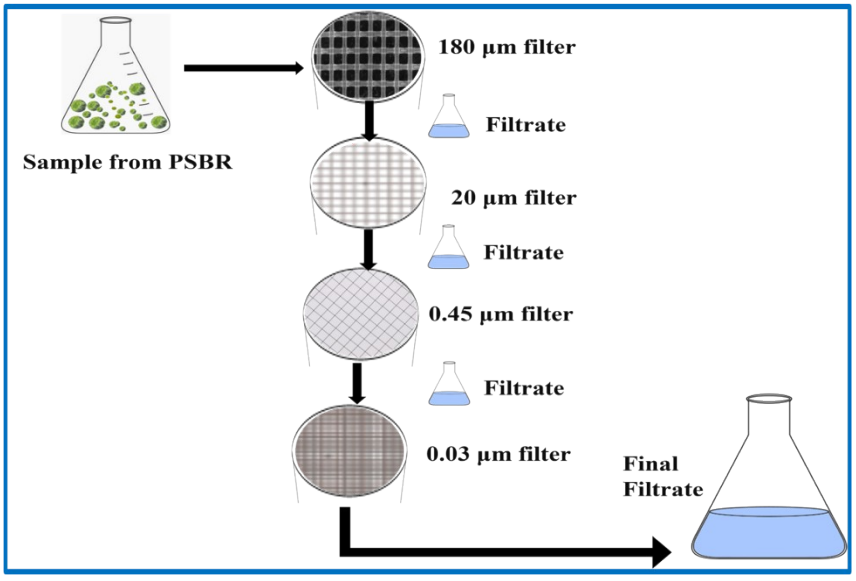

Fig. S1: Experimental setup for particle association and size fraction of pathogen indicators in the photobioreactors.

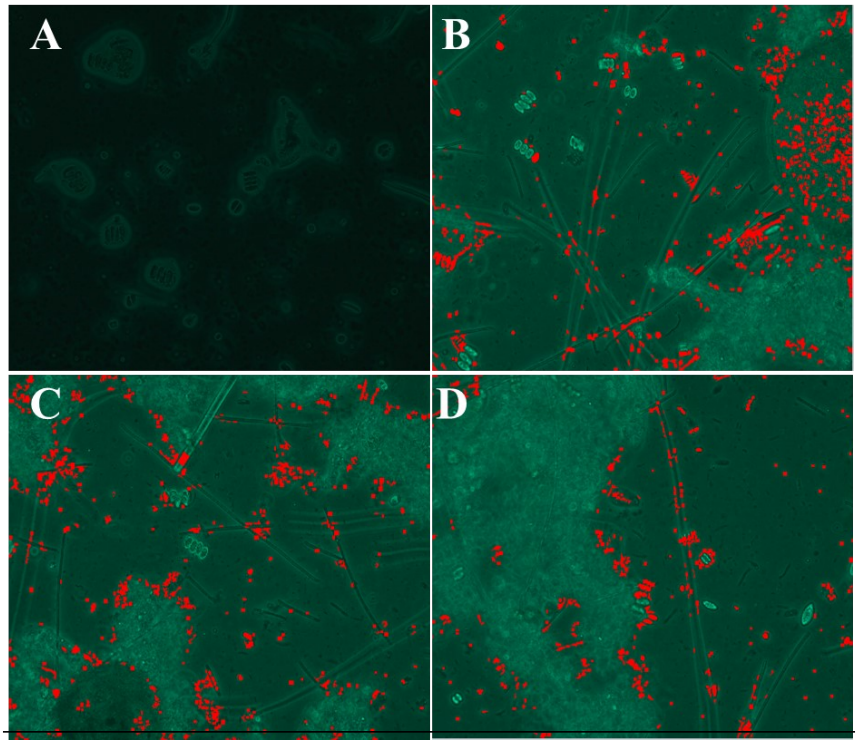

Fig. S2. Fluorescence microscopy images showing evidence of ROS production in algal-bacterial communities from (A) Negative control (without DCFH-DA) (B) Seed (C) PSBR-L (D) PSBR-H after incubation with DCFH-DA. Red dots refer to the presence of ROS.

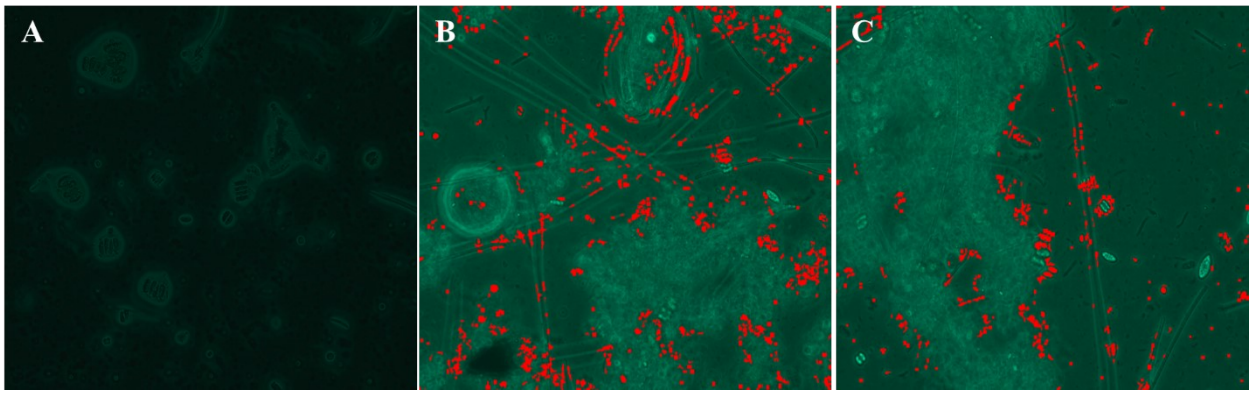

Fig. S3. Fluorescence microscopy images reveal ROS production in algal-bacterial communities from (A) Negative control (without DCFH-DA) (B) Algae-bacteria in real wastewater (C) Algae-bacteria in synthetic wastewater after incubation with DCFH-DA. The red fluorescent dots in images B and C indicate the presence of ROS.

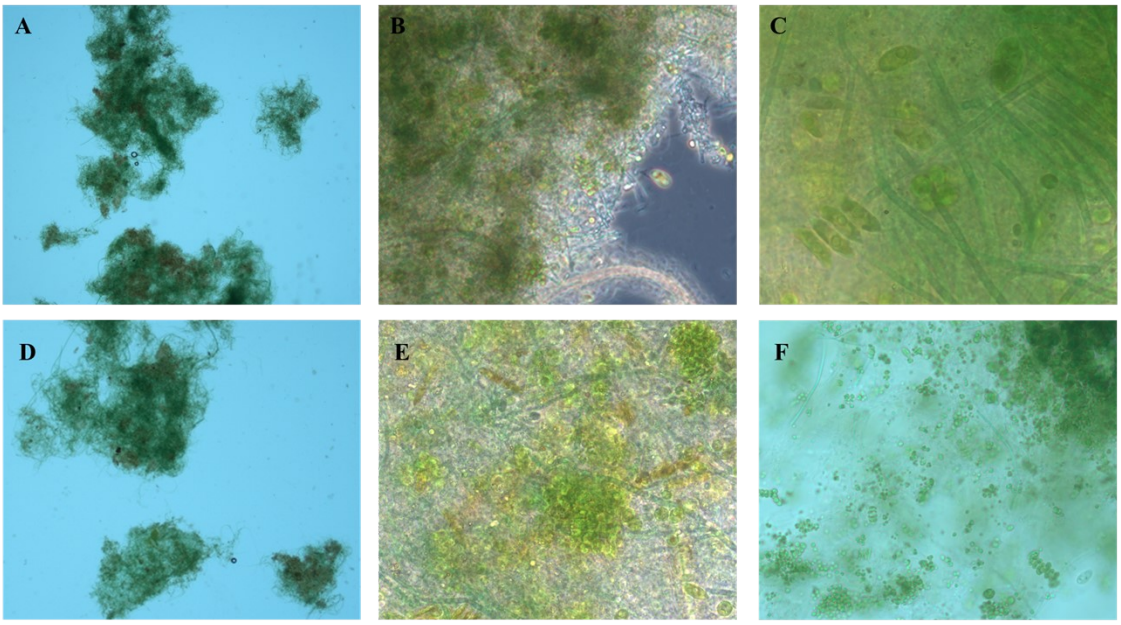

Fig. S4. Microscopic images of algal-bacterial granules from (A, B & C) PSBR-L and (D, E & F) PSBR-H indicate fine particles, bacteria, and other microbes were confined to unicellular and filamentous algae. Images A and D were captured at 10X magnification, B, E and F at 40X magnification, and C at 100X magnification.

References:

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72. M. Pavlovska, I. Prekrasna, I. Parnikoza and E. Dykyi, Soil sample preservation strategy affects the microbial community structure, *Microbes and environments*, 2021, **36**, ME20134.

73. A. Antich, C. Palacín, X. Turon and O. S. Wangensteen, DnoisE: distance denoising by entropy. An open-source parallelizable alternative for denoising sequence datasets, *PeerJ*, 2022, **10**, e12758.