Effects of light irradiation conditions on nitrogen and phosphorus removal in microalgae–bacteria biofilm systems treating low carbon-to-nitrogen wastewater

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Text S1 Flow cytometry testing

1.1. Community abundance

Flow cytometry (FCM) combined with FISH was used to detect bacterial abundance, where Cy5-labeled probes were utilized to reduce the effect of microalgae on the results, as described in our previous studies (Li et al., 2019). The specific circle gate process for microbes was shown in Fig. S2.

1.2. Reactive oxygen species (ROS)

H2DCFDA (2',7' dichlorodihydrofluorescein diacetate) is used as a cell permeability probe to analyze ROS levels. Samples were pretreated according to 1.2 before staining. Subsequently, fluorescent reagents were added to the target cultures with a final concentration of 5 μM. Then, the mixtures were incubated at 37 °C (30 °C for H2DCF-DA) for 30 min and washed three times with 1× PBS, with unstained samples serving as negative controls. Flow cytometry assay results were analyzed with Flow jo V10 software, and results were expressed as mean fluorescence intensity (MFI) based on DCF derivatization.

1.3. Translation activity

Due to the diversity and heterogeneity of activated sludge containing a large number of bacteria, the preparation of activated sludge single-cell suspension is the prerequisite and difficulty for the identification of single cells in activated sludge by FCM analysis. The sample was subject to ultrasonic dispersion treatment at 80 kJ/L to obtain complete free cells, meanwhile avoiding cell damage or death. In addition, the sonication method does not affect cell viability at the Es level. The entire pretreatment

process lasted 5 min. The free cell suspension was filtered on a 20 μm filter membrane to eliminate particles that may block the nozzle of the flow cytometer in the subsequent flow cytometry analysis.

Utilizing BONCAT method to describe translationally active microbial communities in environmental samples has been demonstrated in previous studies on activated sludge, deep-sea and spa sediments, and soil (Reichart et al., 2020; Du and Behrens, 2021). BONCAT was utilized to detect the bacteria with translational activity at different light intensities. Prior to staining, BONCAT was optimized by testing the optimal staining time, amino acid class (L-homohypoglycine (HPG) and L-azidohomoalanine (AHA)) and amino acid reaction concentration (Du and Behrens, 2021). According to the optimized BONCAT, samples were taken at the aerobic stage and incubated with the amino acid analog l-homohypoglycine (HPG). After fluorescent cell staining by click chemistry, BONCAT positivity represents the group of microorganisms with translational activity in the AOA system, while the MFI of the positive region indicates the level of microbial translational activity. After BONCAT staining, the samples were hybridized using fluorescent in situ hybridization (FISH) for the main functional bacterial target genes according to Li et al. (2019) and Du et al. (2021), where probe information and the circle gate process are shown in Table S1, Fig. S3 and Fig. S4, respectively.

1.4. Cell viability, activity and apoptosis assessment

Propidium iodide (PI)/SYBR Green I (SGI), 5(6)-Carboxyfluorescein diacetate (CFDA), Annexin V conjugated to green-fluorescent FITC dye (Annexin V-FITC)/PI

was used to detect cell integrity, esterase activity and early apoptosis, respectively, see Section *2.2* for detailed steps.

Text S2 Calculation method

P release (mg/L) was calculated as the difference between the P concentration at influent and the third anaerobic zone (Eq S1):

$$P\ release = P_{\text{the third anaerobic zone}} - P_{\text{influent}} \quad (S1)$$

Total P uptake (mg/L) was calculated in aerobic (Eq S2) and anoxic zones (Eq S3):

$$P\ uptake = P_{\text{the third anaerobic zone}} - P_{\text{the third aerobic zone}} \quad (S2)$$

$$P\ uptake = P_{\text{the third aerobic zone}} - P_{\text{the last anoxic zone}} \quad (S3)$$

The net P removal efficiency (%) was calculated as the difference between the P concentration in influent and secondary sedimentation tank, divided by the influent P concentration, and multiplied by 100 (Eq S4):

$$P\ removal = (P_{\text{influent}} - P_{\text{the secondary sedimentation tank}}) / P_{\text{influent}} \times 100 \quad (S4)$$

The NH_4^+-N removal efficiency (%) was calculated as the difference between the NH_4^+-N concentration in influent and the secondary sedimentation tank, divided by the influent NH_4^+-N concentration, and multiplied by 100 (Eq S5).

$$NH_4^+\text{-}N\ removal = (NH_4^+\text{-}N_{\text{influent}} - NH_4^+\text{-}N_{\text{the secondary sedimentation tank}})/NH_4^+\text{-}N_{\text{influent}} \times 100 \quad (S5)$$

The total inorganic N (TIN) removal efficiency (%) was calculated as the difference between the influent NH_4^+-N concentration and the concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N in the secondary sedimentation tank, divided by the influent NH_4^+-N concentration, and multiplied by 100 (Eq S6):

$$\text{TN removal} = (NH_4^+\text{-N}_{\text{influent}} - NH_4^+\text{-N}_{\text{the secondary sedimentation tank}} - NO_3^-\text{-N}_{\text{the secondary sedimentation tank}} - NO_2^-\text{-N}_{\text{the secondary sedimentation tank}})/NH_4^+\text{-N}_{\text{the secondary sedimentation tank}} \times 100 \quad \text{(S6)}$$

Text S3 Chlorophyll extraction and quantification

Chlorophyll content in the cultures was determined by extracting chlorophylls from the biomass pellet obtained from 4 mL of reactor sample. Initially, samples were centrifuged at 8000 × g for 5 minutes, after which the supernatant was discarded. The resulting pellet was stored at –20 °C until further analysis. For chlorophyll extraction, 4 mL of 95% (v/v) ethanol was added to the pellet. The mixture was thoroughly vortexed and incubated overnight at room temperature in darkness to prevent chlorophyll degradation. Following incubation, the samples were centrifuged again at 8000 × g for 5 minutes to separate the biomass from the extract.

The absorbance of the resulting supernatant was measured using an Ultrospec 2100 pro (Amersham) spectrophotometer. Chlorophyll concentration was then calculated following the method described by Lichtenthaler (1987), using the following equation:

$$C_{a+b}\ (\text{mg/mL}) = 5.24 \times A_{664.2} + 22.24 \times A_{648.6} \quad \text{(S7)}$$

where C_{a+b} represents the combined concentrations of chlorophyll a and b, and $A_{664.2}$ and $A_{648.6}$ denote the absorbance values at the respective wavelengths.

Table S1

FCM-FISH and BONCAT-FISH probe sequences.

Probe types	Target microbe	Probe sequence
NIT3	*Nitrobacter*	CCTGTGCTCCATGCTCCG
Ntspa662	*Nitrospira*	GGAATTCCGCGCTCCTCT
Ntoga122	*Nitrotoga*	TCCGGGTACGTTCCGATAT
NSO 190	*Nitrosomonas*	CGATCCCCTGCTTTTCTCC
PAO651	Most of PAOs	CCCTCTGCCAAACTCCAG
PAO462		CCGTCATCTACWCAGGGTATTAAC
PAO846		GTTAGCTACGGCACTAAAAGG
Dech453	*Dechloromonas*	GGGTATTCACCCATGCGA
Dech443		ACC CATGCATTTTCT TCC CGG
Bet135	*Competibacter*	ACGTTATCCCCCACTCAATGG
GAOQ431		TCCCCGCCTAAAGGGCTT
GAOQ989		TTCCCCGGATGTCAAGGC
TFO_DF218 522R	*Defluviicoccus*	GAAGCCTTTGCCCCTCAG
TFO_DF618		GCCTCACTTGTCTAACCG
DF988		GATACGACGCCCATGTCAAGGG
DF1020		CCGGCCGAACCGACTCCC
Acc-I-444	*Accumulibacter* I	CCCAAGCAATTTCTTCCCC
Acc-II-444	*Accumulibacter* II	CCCGTGCAATTTCTTCCCC

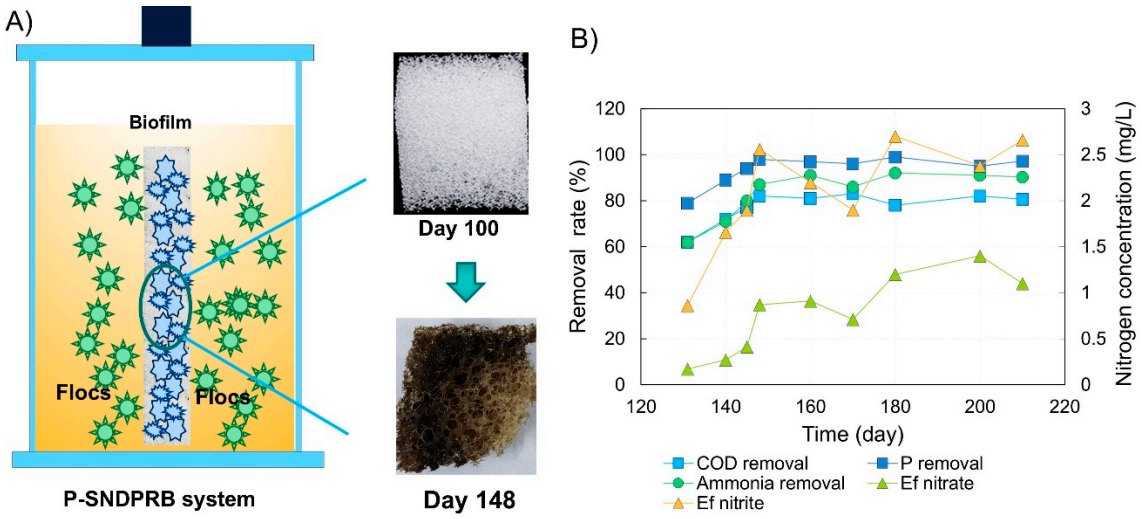

Fig. S1 Changes in filler profile during biofilm formation (A) and biofilm performance on nutrient removal (B) in the P-SNDPRB system.

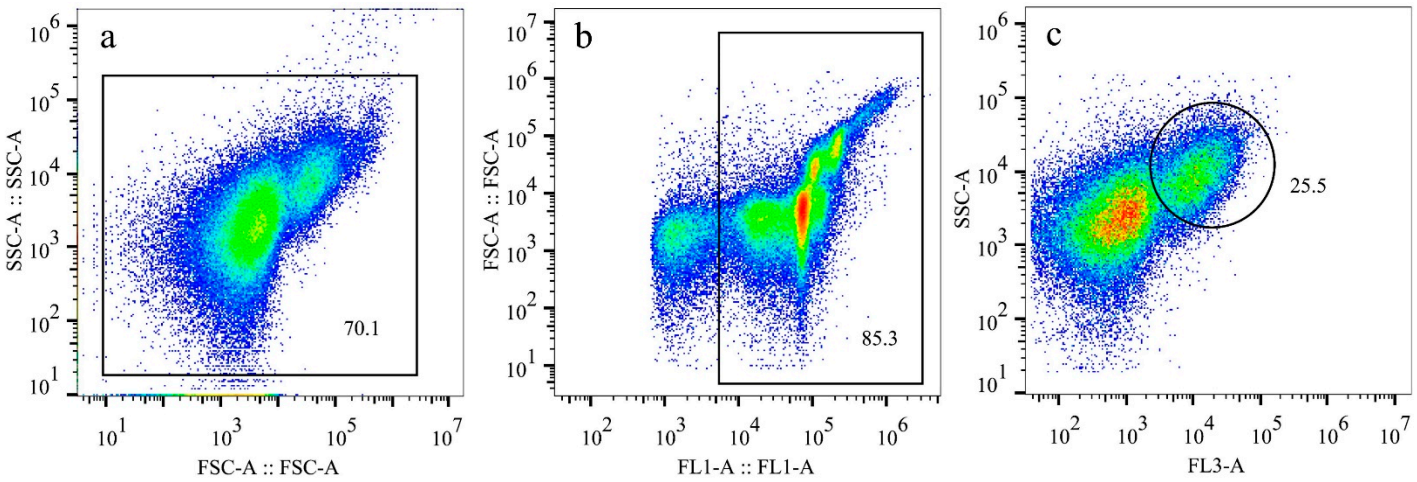

Fig. S2 Circle gate process for sample detection by FCM-FISH; a. exclude tiny cell debris; b. exclude non-DNA large particles; c. circle positive regions

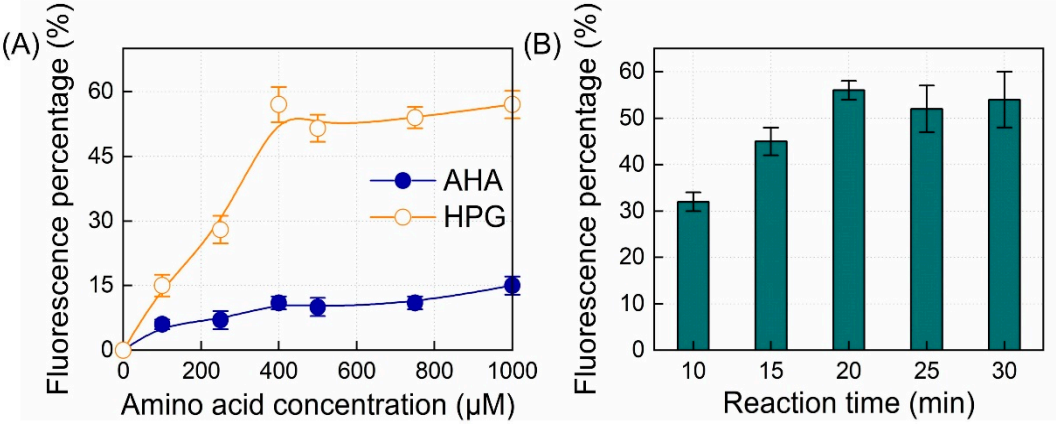

Fig. S3 The response of amino acid type and concentration (A) and incubation time (B) to fluorescent staining in BONCAT.

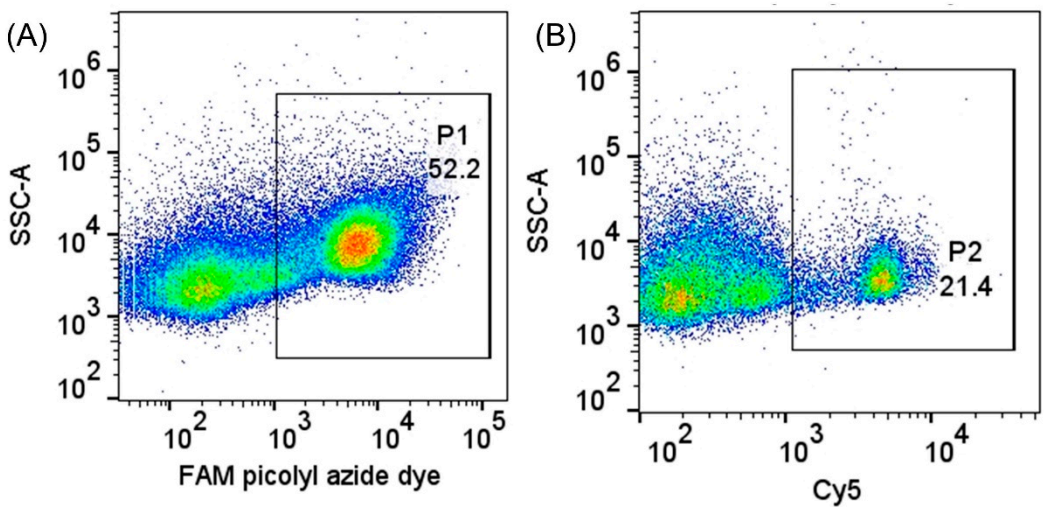

Fig. S4 BONCAT-FISH circle gate process. P1 represents the percentage of BONCAT+ bacteria in total bacteria (A); P2 represents the percentage of specific functional bacteria in total BONCAT+ bacteria (B).

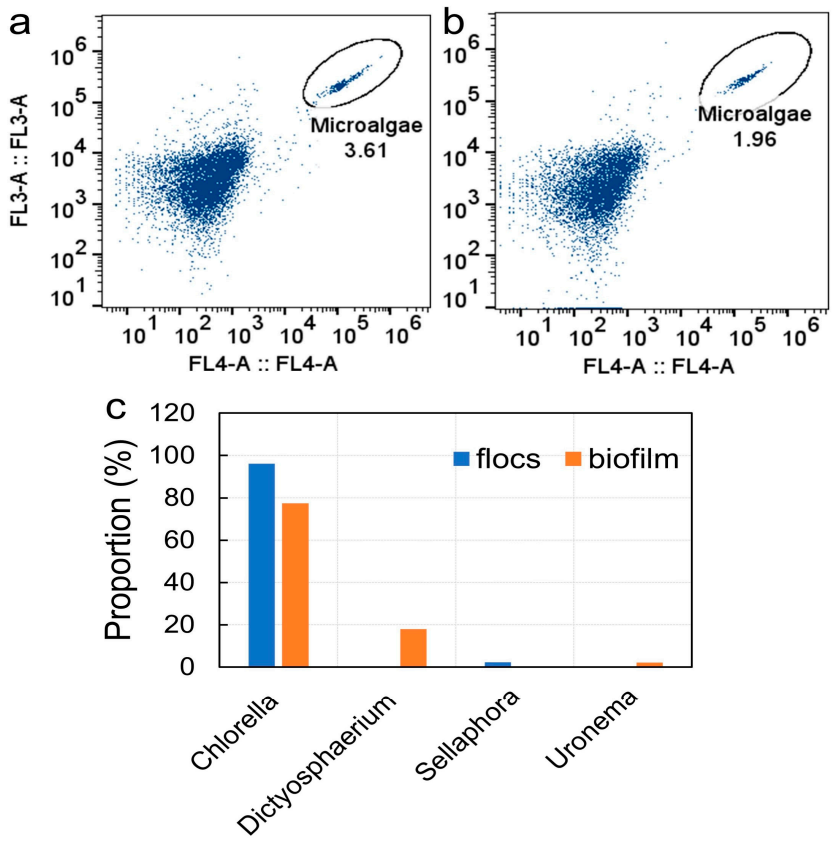

Fig. S5 Population abundance (a. flocs and b. biofilms) and composition (c) of microalgae in the P-SNDPRB system.

References

Du, Z., Behrens, S.F., 2021. Tracking de novo protein synthesis in the activated sludge microbiome using BONCAT-FACS. Water Res. 117696.

Li, C., Zeng, W., Li, N., Guo, Y., Peng, Y., 2019. Population Structure and Morphotype Analysis of "Candidatus Accumulibacter" Using Fluorescence In Situ Hybridization-Staining-Flow Cytometry. Appl. Environ. Microb. e02943-189.