

Supplementary Material

Unraveling the role of formate in improving nitrogen removal via coupled partial denitrification-anammox

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Table S1. Primers for RT-qPCR

Primer name	Primer sequence (5' → 3')	Target	Ta (°C)	Length	Ref
narG-f	TCGCCSATYCCGGCSATGTC	narG	60	173	1
narG-r	GAGTTGTACCAGTCRGCSGA				
nirSCd3aF	AACGYSAAGGARACSSG	nirS	60	407	2
nirSR3cd	GASTTCGGRTGSGTCTTSAY				
HZOQPC	AAGACNTGYCAYTGGGGWA	hzo	53	224	3
HZOQPC	GACATACCCATACTKGTRTA				
Amx368F	TTCGCAATGCCCGAAAGG	AnAOB	55	453	4
Amx820R	AAAACCCCTCTACTTAGTGC	16S rDNA			
universal3	CCTACGGGAGGCAGCAG	Eubacteria	60	193	5
universal5	TTACCGCGGCTGCTGGCA	16S rDNA			

Table S2. Fluorescence *in situ* hybridization probes

Probe	5' modification	Sequence 5' → 3'	Target group	Ref
Amx568	Alexa fluor 568*	CCTTTCGGGCATTGCGAA	AnAOB	4
PRO405	Alexa fluor 405*	TGCGATTTCTTCCCGGCC	Rhodocyclaceae	6

Table S3. Detailed information of key genes involved in amino acid biosynthesis

Amino acid	Gene	Enzyme
Ala	asdA	aspartate 4-decarboxylase
	alaA	alanine-synthesizing transaminase
	argB	acetylglutamate kinase
	argC	N-acetyl-gamma-glutamyl-phosphate reductase
	argD	acetylmethionine/N-succinylmethionine aminotransferase
Arg	argE	acetylmethionine deacetylase
	argJ	glutamate N-acetyltransferase / amino-acid N-acetyltransferase
	argFI	ornithine carbamoyltransferase
	argG	argininosuccinate synthase
	argH	argininosuccinate lyase
Asn	asnA	aspartate-ammonia ligase
	asnB	asparagine synthase (glutamine-hydrolysing)
Asp	aspC	aspartate aminotransferase
Cys	cysK	cysteine synthase
	cth	cystathionine gamma-lyase

	mccB	cystathionine gamma-lyase
Glu	gltB	glutamate synthase (NADPH) large chain
	gltD	glutamate synthase (NADPH) small chain
Gly	glyA	glycine hydroxymethyltransferase
	hisG	ATP phosphoribosyltransferase
His	hisA	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase
	hisB	imidazoleglycerol-phosphate dehydratase
	hisC	histidinol-phosphate aminotransferase
	hisD	histidinol dehydrogenase
	hisH	imidazole glycerol-phosphate synthase subunit
	hisI	phosphoribosyl-AMP cyclohydrolase
Ile	ilvD	dihydroxy-acid dehydratase
	ilvC	ketol-acid reductoisomerase
Leu	leuA	2-isopropylmalate synthase
	leuB	3-isopropylmalate dehydrogenase
	leuC/D	3-isopropylmalate/(R)-2-methylmalate dehydratase large subunit
Lys	lysA	diaminopimelate decarboxylase
	lysAC	bifunctional diaminopimelate decarboxylase / aspartate kinase
Met	metC	cysteine-S-conjugate beta-lyase
	metH	5-methyltetrahydrofolate--homocysteine methyltransferase
Phe	pheC	cyclohexadienyl dehydratase
	pheA2	prephenate dehydratase
	hisC	histidinol-phosphate aminotransferase
	proB	glutamate 5-kinase
Pro	proA	glutamate-5-semialdehyde dehydrogenase
	proC	pyrroline-5-carboxylate reductase
	serA	D-3-phosphoglycerate dehydrogenase
Ser	serB	phosphoserine phosphatase
	hom	homoserine dehydrogenase
Thr	thrB	homoserine kinase type II
	thrC	threonine synthase
Trp	trpE/G	anthranilate synthase component I
	trpC	indole-3-glycerol phosphate synthase
	trpA/B	tryptophan synthase alpha chain
Tyr	phhA	phenylalanine-4-hydroxylase
Val	ilvE	branched-chain amino acid aminotransferase

Text S1: FISH procedure

The fixed sample was added to 5 ml of $1 \times$ PBS solution containing 25 μ L of Tween 20, vortex for 30 min⁷, and then ultrasonicated for 5 min at 2% power with a working frequency of 1 s and 4 s intervals to obtain a high-density dispersed biomass sample⁸. The dispersed sample was then centrifuged at 800 rpm for 10 min to get the supernatant, at 3000 rpm for 10 min to get another supernatant, and 13000 rpm for 5 min to collect the precipitate as a clean biomass sample. 50%, 80% and 98% ethanol solutions were then added to the centrifuge tube containing the sample, soaked for 3 minutes respectively, and finally dried in the air. 3mL hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 35% formamide, pH=7.2) was prepared and mixed with 1 mL 10 nmol/mL Amx568 and 1 mL 10 nmol/mL PRO405. Afterwards, the centrifuge tube containing the sample was added 50uL hybridization buffer and incubated in a dark 48°C incubator for 8 hours. The washing buffer (338 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, pH 7.2) was preheated to 48°C in advance. The sample was then centrifuged after hybridization with the removal of the supernatant and the washing buffer was added. Incubated at 48°C for 30 minutes, the supernatant of the buffered sample was then removed by centrifugation. With differential centrifugation and final 5 minutes at 13000 rpm, 50 uL of the supernatant was retained and vortex mixed.

Text S2: Economic cost calculations

The total treatment cost is mainly divided into the cost of pharmaceutical dosing, sludge treatment and other costs. In order to simplify the calculation, it is assumed that the treated water volume is 1000m³/d and the water contains 20mg/L NH₄⁺-N and 20mg/L NO₃⁻-N. Other costs (such as equipment construction costs, management and operation costs) are set unchanged, and only the difference between the cost of chemical dosing and sludge treatment costs is considered.

In terms of agent dosing cost, the market price of formate is \$330/ton (98% purity), the dosing ratio is COD/NO₃⁻-N=1.5, and each g of formate can provide 0.235 g COD. The market price of sodium acetate is \$375/ton (60% purity), the dosing ratio

is COD/NO₃⁻-N=3, and each g of sodium acetate can provide 0.78 g COD.

Therefore, the cost of formate dosing is:

$$1000\text{m}^3/\text{d} * \frac{20\text{mg}/\text{L} * 1.5}{0.235} * \frac{\$330/\text{ton}}{98\%} = \$ 42.99/\text{d}$$

And the cost of acetate dosing is:

$$1000\text{m}^3/\text{d} * \frac{20\text{mg}/\text{L} * 3}{0.78} * \frac{\$375/\text{ton}}{60\%} = \$ 48.08/\text{d}$$

In terms of the cost of sludge treatment, the market cost of sludge treatment is \$20-80/ton, and \$45/ton is taken to calculate.

The Yield value for AnAOB is 0.098 mol biomass/mol NH₄⁺-N, where biomass is calculated as CH₂O_{0.15}N_{0.5}⁹. Then the mud production of AnAOB is:

$$1000\text{m}^3/\text{d} * \frac{20\text{mg}/\text{L}}{14\text{g}/\text{mol}} * 0.098 * 23.4\text{g}/\text{mol} = 3.27\text{kg}/\text{d}$$

The Yield value of denitrifying bacteria (considering only nitrate to nitrite) under the action of formate is 0.025 mol biomass/mol HCOO⁻¹⁰, where biomass is calculated as C₅H₇NO₂. Then the mud production of denitrifying bacteria under the action of formate is:

$$1000\text{m}^3/\text{d} * \frac{20\text{mg}/\text{L} * 1.5}{0.235 * 68\text{g}/\text{mol}} * 0.025 * 113\text{g}/\text{mol} = 5.30\text{kg}/\text{d}$$

The yield value of denitrifying bacteria (considering only nitrate to nitrite) under the action of acetate is 0.13 mol biomass/mol CH₃COO⁻^{10,11}, where biomass is calculated as C₅H₇NO₂. Then the mud production of denitrifying bacteria under the action of acetate is:

$$1000\text{m}^3/\text{d} * \frac{20\text{mg}/\text{L} * 3}{0.78 * 82\text{g}/\text{mol}} * 0.13 * 113\text{g}/\text{mol} = 13.78\text{kg}/\text{d}$$

The cost of sludge treatment under the action of sodium formate is \$0.38/d, and the cost of sludge treatment under the action of sodium acetate is \$0.77/d.

Comprehensively, using formate can save 11.2% of the cost compared with acetate (\$43.37 vs. \$48.85), which is mainly reflected in the saving of the cost of agent dosage.



Fig. S1. The lab-scale anammox moving bed biofilm reactor

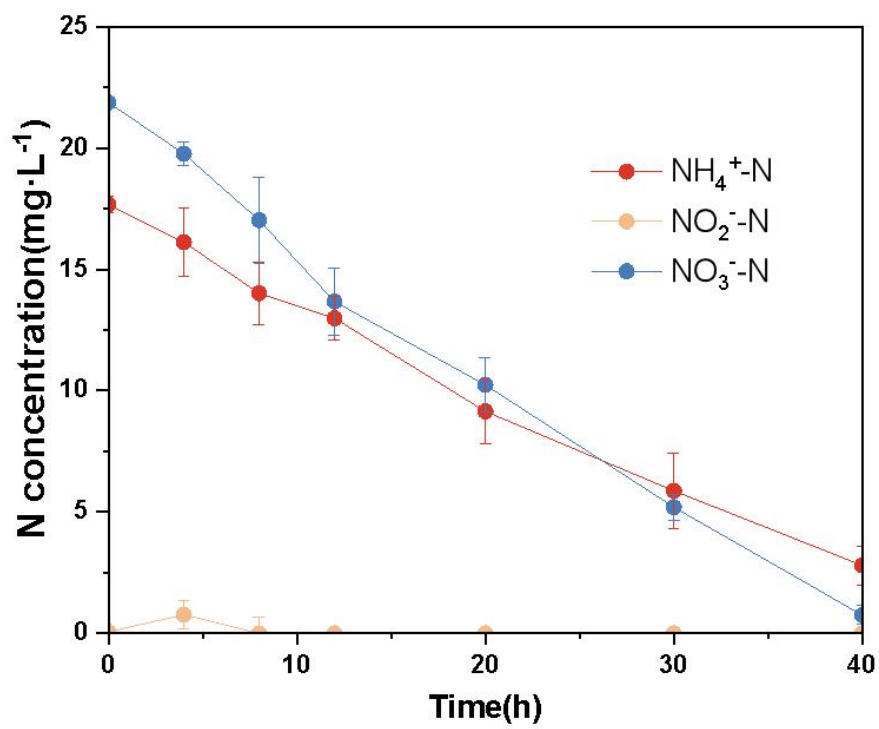


Fig. S2. Changes in nitrogen concentrations during activity tests for FISH-NanoSIMS with formate-driven PD-A biofilm samples

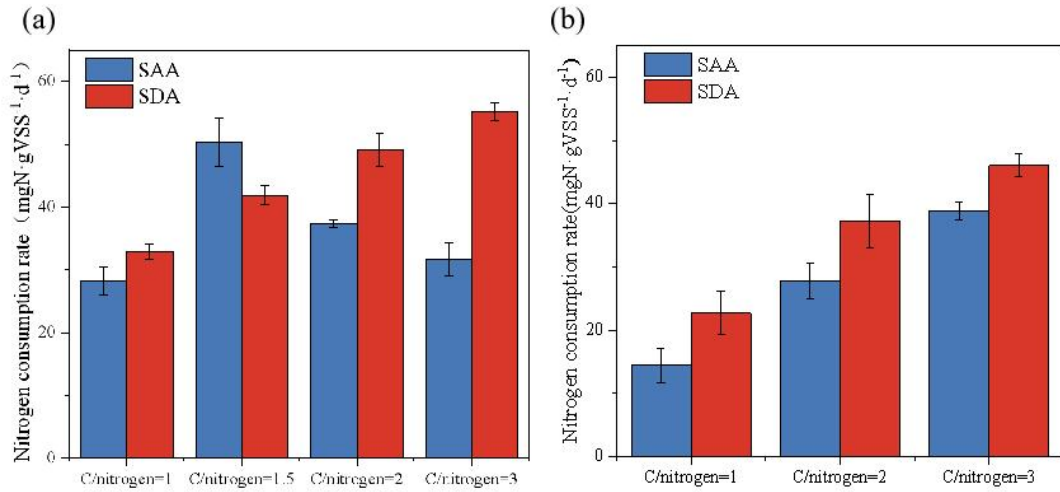


Fig. S3. Specific anammox (SAA) and denitrification (SDA) activities of biofilm samples in phase I (Day 50) under different dosage of (a) formate and (b) acetate by addition of ammonium and nitrate. Error bars represent standard deviations from biological triplicate.

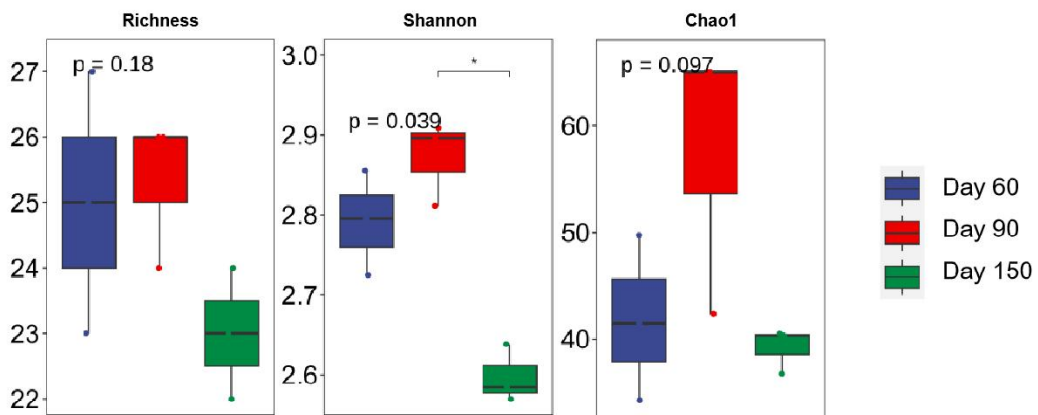


Fig. S4. Alpha diversity of the biofilm community in phase I (day 60) and II (day 90 and 150).

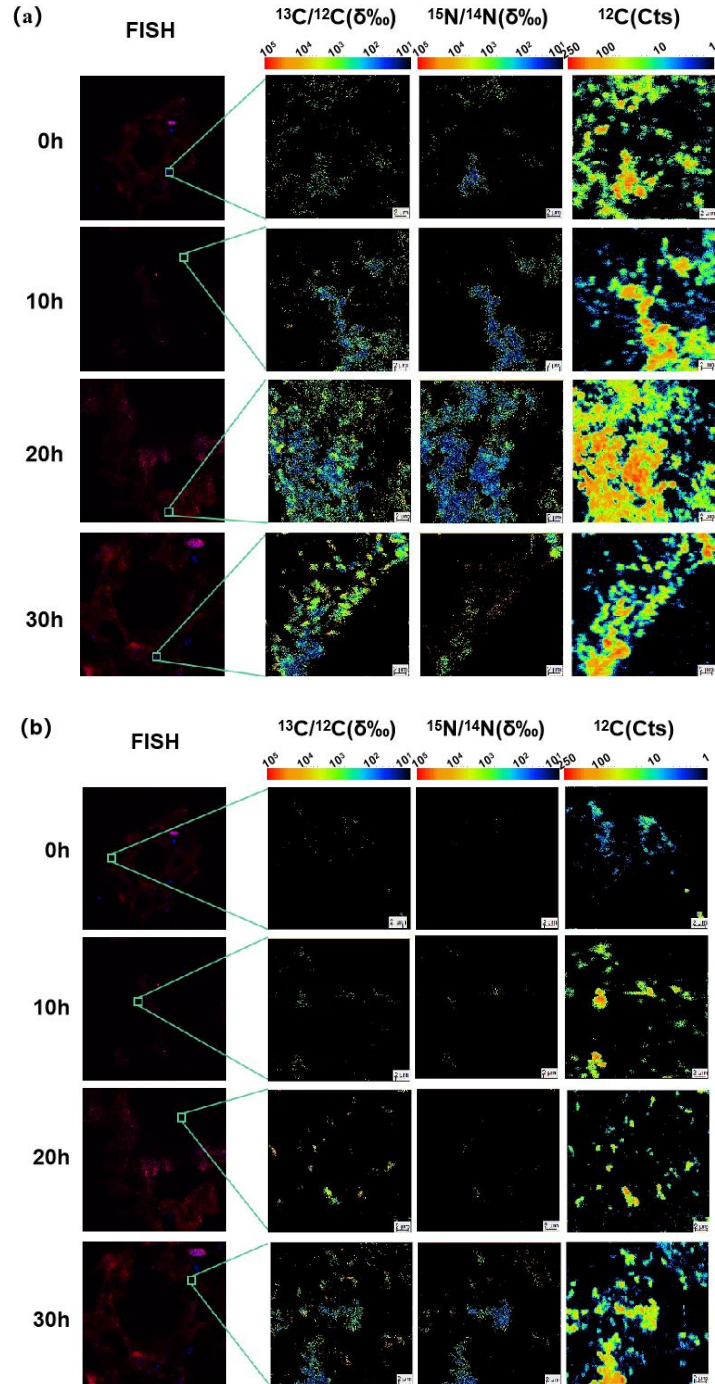


Fig. S5. FISH-NanoSIMS indicated ^{13}C -formate and ^{15}N -nitrate uptake by (a) *Desulfobacillus*, and (b) other bacteria at the single-cell level. Representative FISH images were taken for samples collected at $t=0\text{h}$, 10h , 20h , and 30h during incubation (left column). Small squares denote *Desulfobacillus*- or other bacteria-enriched regions, which are enlarged in the NanoSIMS images (right columns). Scale bar: 2 μm ; Color bar: carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$), nitrogen isotope ratios ($^{15}\text{N}/^{14}\text{N}$), and ^{12}C counts for *Desulfobacillus* or other bacteria.

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