

Supplementary Information

Propagation of side-stream partial nitrification–anammox disturbance to mainstream wastewater greenhouse gas emissions

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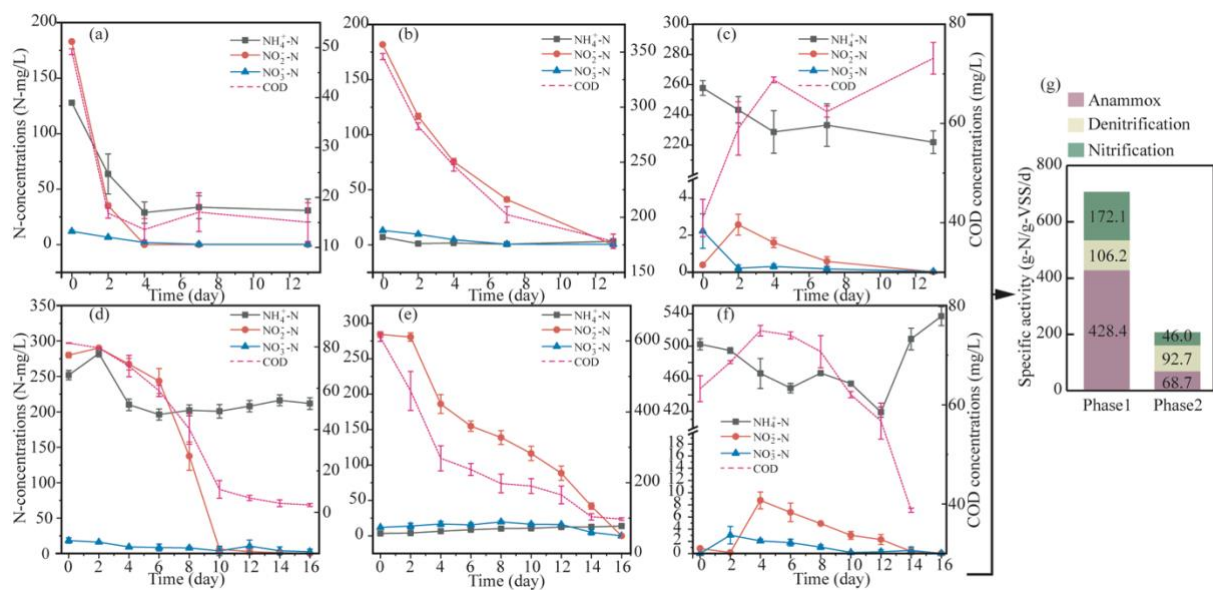


Fig. S1 Specific activity tests. (a)~(c): activity of anammox, denitrification, and nitrification in Phase1, respectively. (d)~(f): activity of anammox, denitrification, nitrification in Phase2, respectively. (g) comparison of specific activity test between two phases.

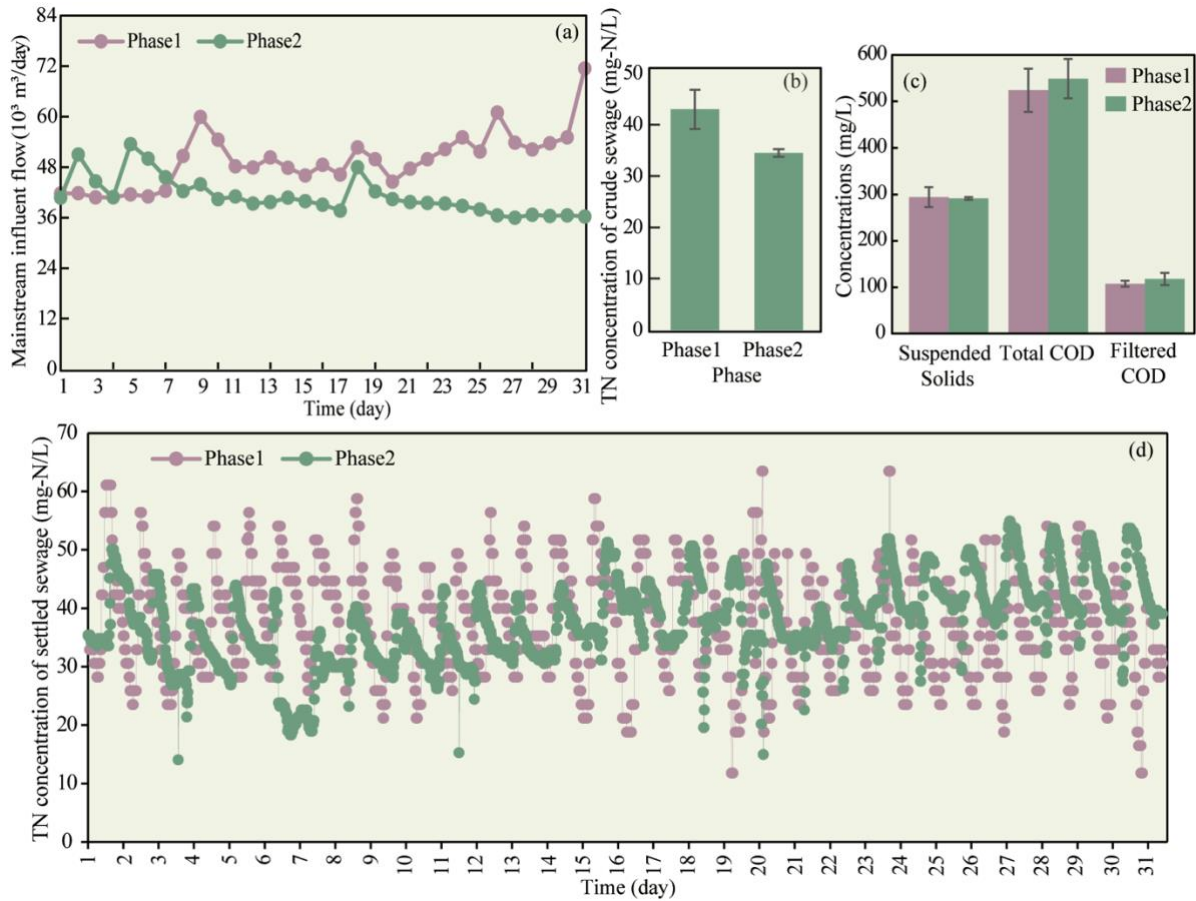


Fig. S2 Nitrogen concentration and flow rate of crude sewage and mainstream influent. (a) Mainstream influent flow. (b) TN concentration of crude sewage in two phases. (c) Suspended solids, Total COD, filter COD concentration of crude sewage. (d) TN concentration of settled sewage in two phases. For each phase in (a) and (d), 30 days of data are presented. Data averaged over one month were used for comparison between two phases.

At the kingdom level, the relative abundance of archaea decreased from $27.4 \pm 5.9\%$ in Phase1 to $18.0 \pm 4.3\%$ in Phase2 (Fig. S3) in the AD unit. In contrast, the PN/A tank increased from $24.3 \pm 8.0\%$ in Phase1 to $28.7 \pm 0.9\%$ in Phase2). In the Anoxic and Aerobic tanks, archaea abundance in Phase2 was 8.8-fold and 11.1-fold higher, respectively, compared to Phase1. Clearly, the AD unit and disturbed PN/A provided a source of archaea population to the mainstream units.

At the phylum level, the AD unit was dominated by Firmicutes, Euryarchaeota, and Atribacterota (Fig. S3). From Phase1 to Phase2, Firmicutes increased ($43.6 \pm 5.3\%$ to $52.0 \pm 4.8\%$), while Euryarchaeota ($27.4 \pm 6.0\%$ to $18.0 \pm 4.3\%$) and Atribacterota ($20.0 \pm 3.0\%$ to $16.7 \pm 3.7\%$) declined. In the PN/A tank, Planctomycetes and Proteobacteria declined sharply from $17.9 \pm 2.2\%$ and $13.1 \pm 5.1\%$ in Phase1 to $0.57 \pm 0.03\%$ and $7.2 \pm 2.2\%$ in Phase2, respectively. Conversely, in the Anoxic and Aerobic tanks, Proteobacteria increased by $16.1 \pm 3.1\%$ and Nitrospirae by $17.8 \pm 3.3\%$ from Phase1 to Phase2.

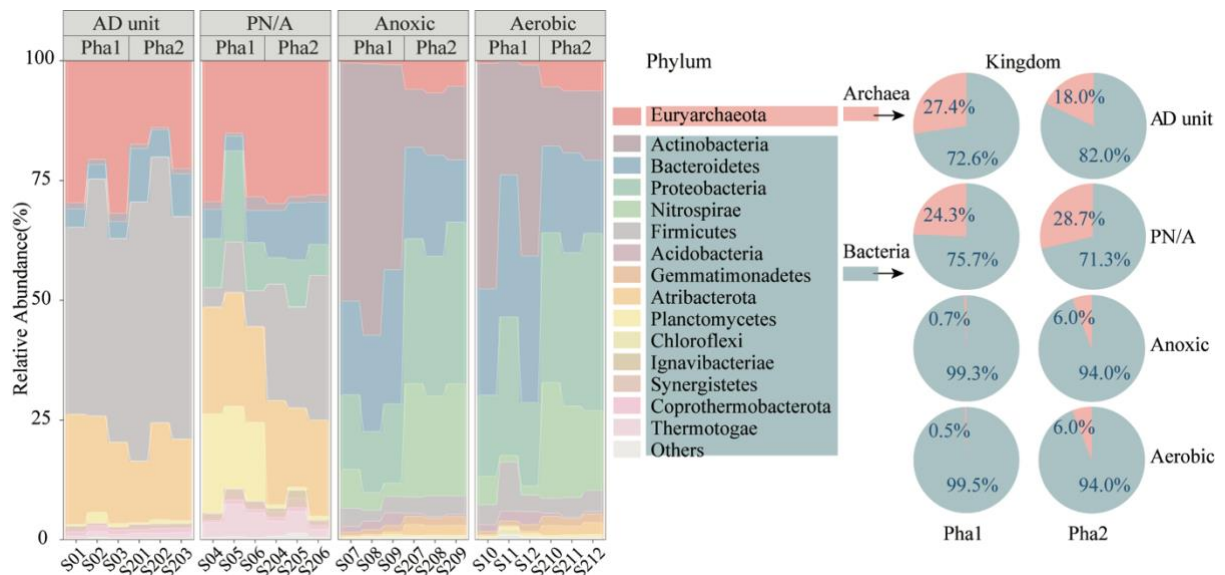


Fig. S3 Microbial community composition at kingdom and phylum levels across treatment units and operational phases. The stacked bar plot shows the relative abundance of dominant microbial phyla in the AD unit, PN/A tank, Anoxic tank, and Aerobic tank during Phase1

41 (Pha1) and Phase2 (Pha2). Each bar represents an individual sample, and taxa with low
42 relative abundance are grouped as “Others”. The pie charts show the relative proportions of
43 Archaea and Bacteria at the kingdom level in each treatment unit and phase. Pha1: Phase 1;
44 Pha2: Phase 2.

45 For functional profiling, predicted genes were annotated against the KEGG database, and
46 assigned to metabolic pathways at levels 1-3 based on KEGG Orthologs (KOs) (Fig. S4). The
47 relative abundance of each pathway was calculated as the sum of RPKM-normalized
48 abundances of KOs assigned to that pathway, divided by the total RPKM-normalized
49 abundance of all annotated KOs within each sample and domain (bacteria or archaea), and
50 expressed as a percentage. In the AD unit, reactions by archaea decreased significantly from
51 24.4% in Phase1 to 15.7% in Phase2 ($p < 0.05$), while bacteria (>75%) dominated in both phases
52 (Fig. S4a). In contrast, archaeal reactions increased in the downstream three tanks during
53 Phase2, consistent with the observed shifts in microbial community composition. At KEGG
54 level 1, Metabolism was the most abundant group (>95%) in all tanks for both archaea and
55 bacteria (Fig. S4b&c). At level 2, the top pathways included Energy metabolism, Carbohydrate
56 metabolism, and Metabolism of cofactors and vitamins (Fig. S4d&e). These pathways are
57 critical for organic matter degradation, energy production, and nitrogen metabolism, reflecting
58 the robust metabolic capabilities of the microbial communities in the WWTP.¹

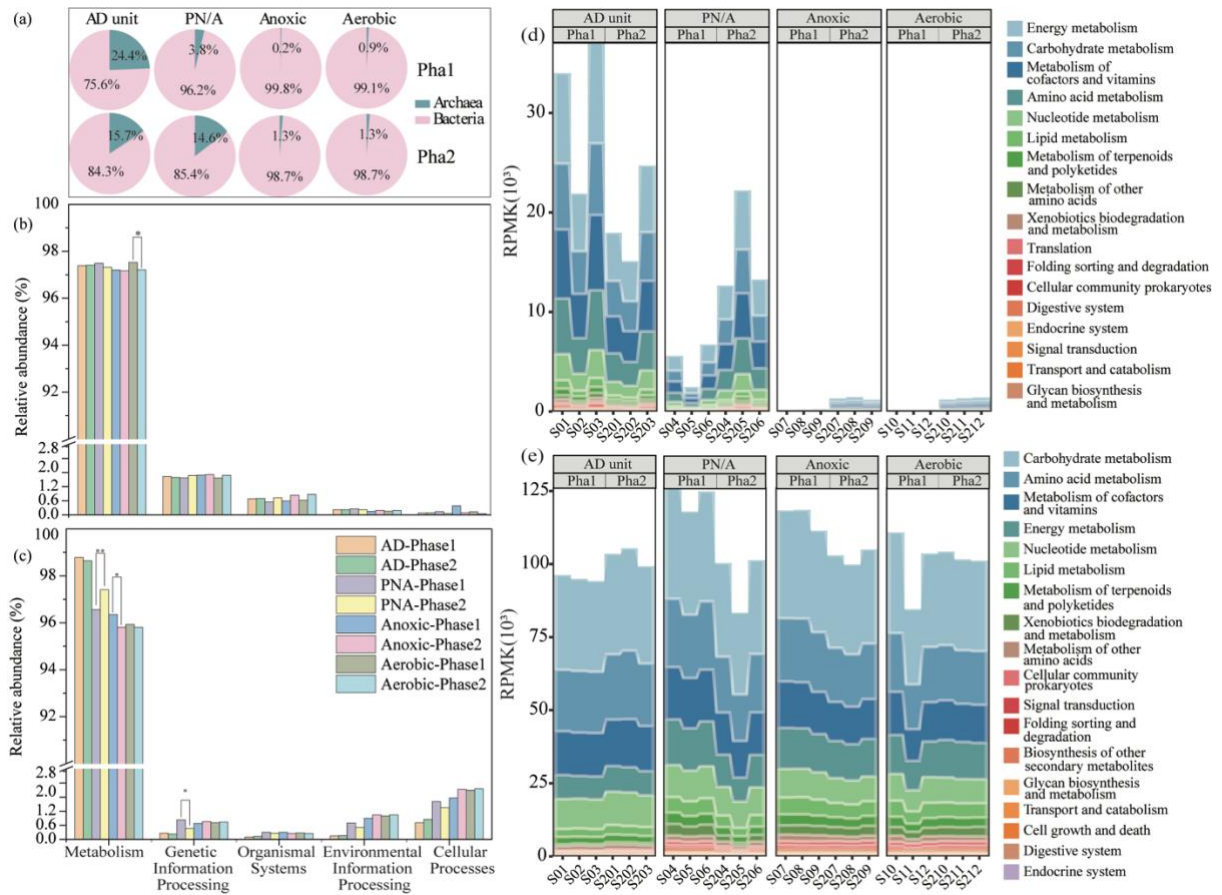


Fig. S4 Relative abundance of KEGG metabolic pathways at level-1 and level-2. (a) carried out by archaea and bacteria. (b) at level-1 carried out by archaea. (c) at level-1 carried out by bacteria. (d) at level-2 carried out by archaea. (e) at level-2 carried out by bacteria. Statistical comparisons were based on n=3 independent samples per phase.

70 At level 3, for archaea, Methane metabolism (KEGG:00680) pathways involved in methane
71 metabolism dominated in the AD unit (17.18% - 19.82%) (Fig. S5a), aligning with its role in
72 CH₄ production.² In Phase2, methane metabolism increased in the downstream three tanks,
73 suggesting enhanced methanogenic potential and CH₄ emissions. Conversely, oxidative
74 phosphorylation and tricarboxylic acid (TCA) cycle activity decreased in the anoxic and
75 aerobic tanks, indicating a shift towards anaerobic metabolism. For bacteria, Oxidative
76 phosphorylation (3.62% - 4.92%, KEGG:00190) and Glycolysis (3.51% - 5.86%,
77 KEGG:00010) were the most abundant KEGG level 3 pathways (Fig. S5b). Compared to Phase
78 1, glycolysis increased in the PN/A tank but decreased in the Anoxic and Aerobic tanks, while
79 nitrogen metabolism decreased in the PN/A tank but increased in the Anoxic and Aerobic tanks,
80 and oxidative phosphorylation decreased in all three tanks in Phase 2. These results indicate a
81 metabolic shift towards anaerobic pathways in the downstream tanks, driven by the disturbed
82 PN/A process and the resulting changes in PN/A effluent composition.³

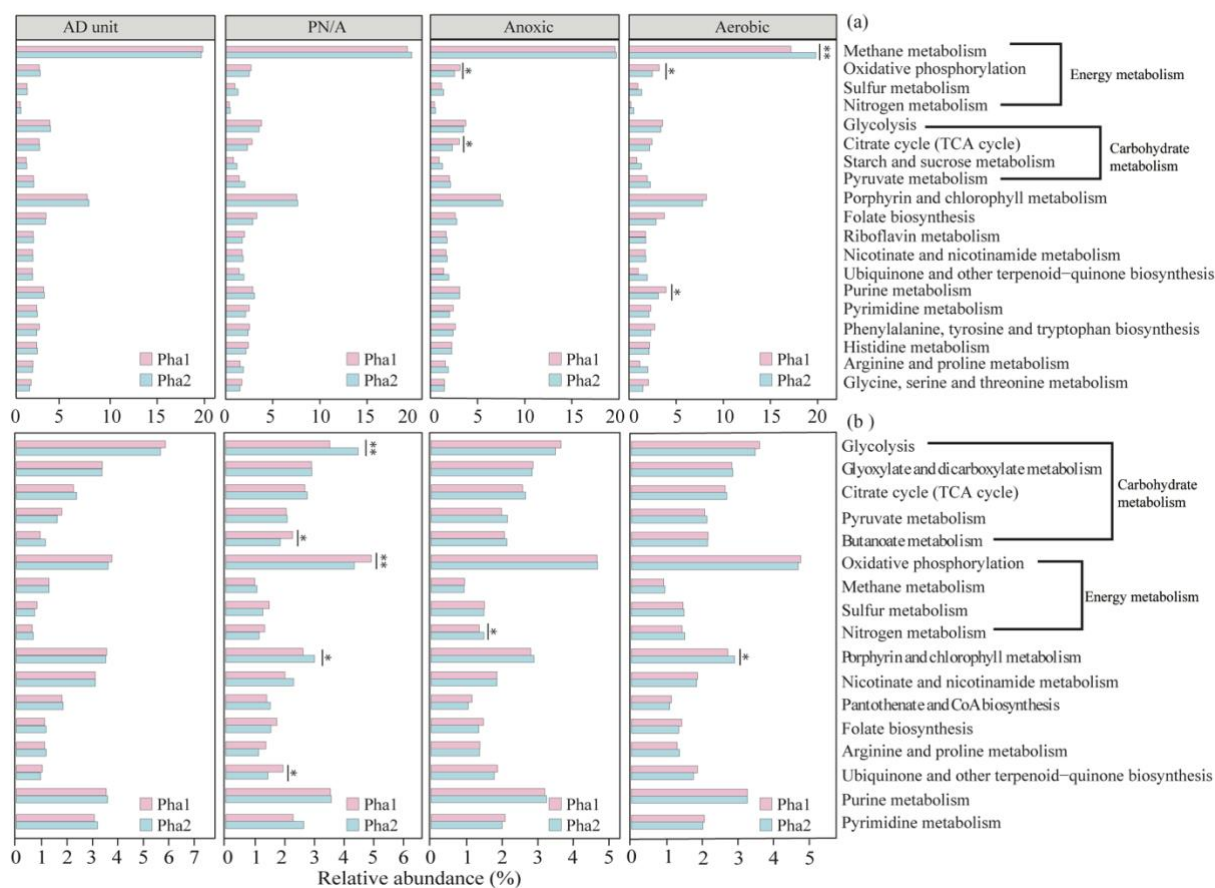


Fig. S5 Relative abundance of KEGG metabolic pathways at level3. (a) carried out by archaea. (b) carried out by bacteria. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Statistical comparisons were based on $n=3$ independent samples per phase.

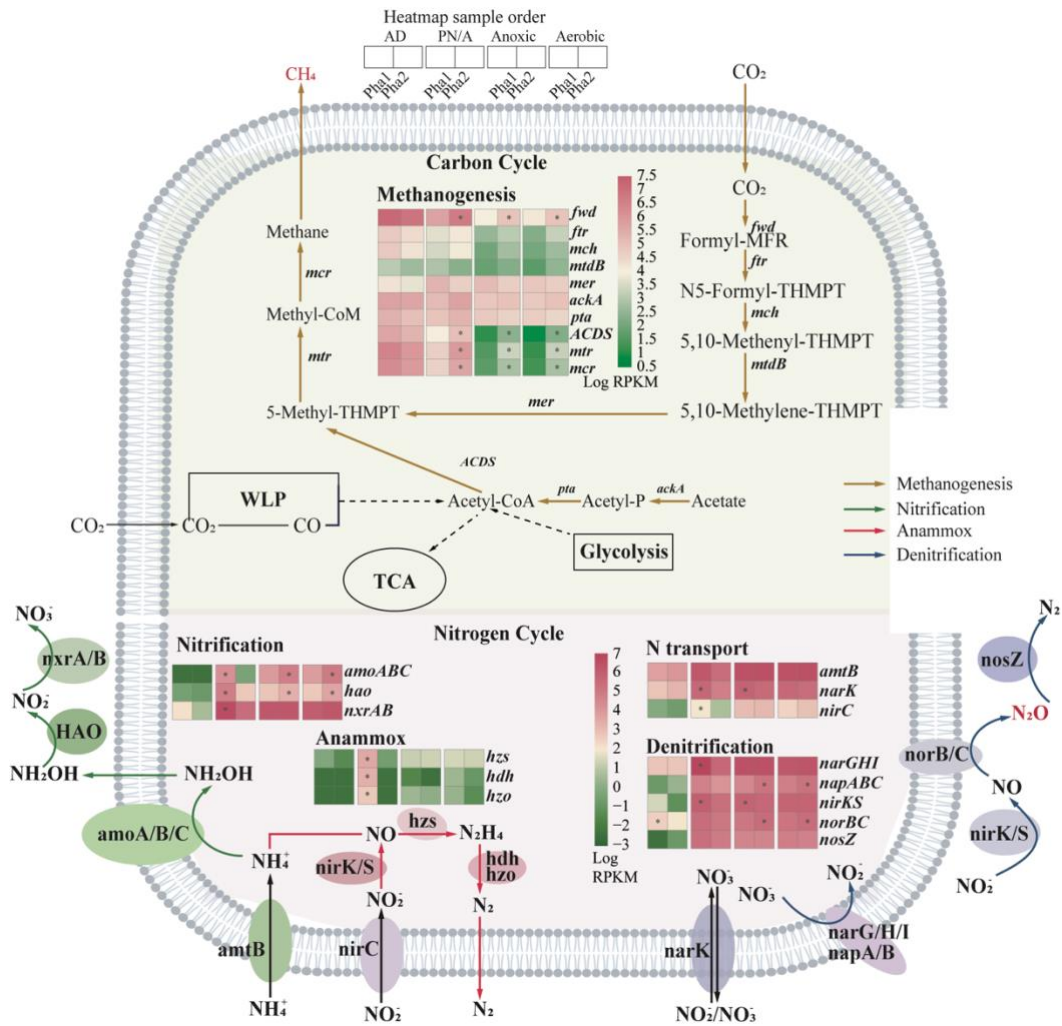
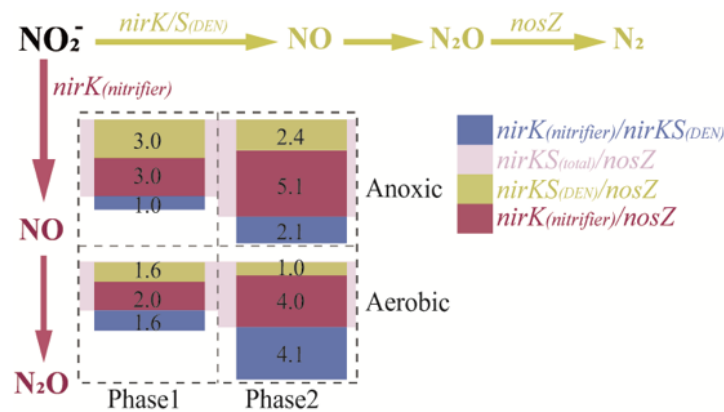


Fig. S6 CH₄ and Nitrogen metabolic pathways and related functional gene abundances. Gene abundance refers to the overall abundance of a given gene across the entire microbial community, rather than within a specific microorganism. The colour key indicates the abundance calculated as PRKM in the log scale.

92 Roothans et al. reported that the ratio between *nirK/S* and *nosZ* in denitrifiers typically remains
 93 relatively stable (0.5 - 1.5) across seasonal changes,⁴ indicating denitrifiers are unlikely to be
 94 the main N₂O producers. Ratios between the abundance of *nirK/S*_(total) (AOB + NOB +
 95 denitrifiers), *nirK/S*_(DEN) (denitrifiers only), and *nirK*_(nitrifier) (AOB + NOB) and the only known
 96 N₂O-reducing enzyme *nosZ* were calculated (Fig. S7; Supplementary Data 4). The ratio of
 97 *nirK/S*_(total) to *nosZ* increased from 5.9 and 3.7 to 7.5 and 5.0, while the ratio of *nirK/S*_(DEN) to
 98 *nosZ* declined from 3.0 and 1.6 in Phase1 to 2.4 and 1.0. Meanwhile, the ratio of *nirK*_(nitrifier) to
 99 *nosZ* increased markedly, from 3.0 and 2.2 in Phase1 to 5.1 and 4.0 in Phase2 in the Anoxic
 100 and Aerobic tanks, respectively. Furthermore, *nirK*_(nitrifier) and *nirK/S*_(DEN) increased from 1.0
 101 and 1.6 in Phase1 to 2.1 and 4.1 in Phase2, suggesting a relatively greater representation of
 102 nitrifier-denitrification genes compared with denitrifier *nirK/S* in Phase2.



104 Fig. S7 Ratios between nitrite consuming enzyme *nirK/S* and N₂O reducing enzyme *nosZ* in
 105 the Anoxic and Aerobic tanks. *nirK*_(nitrifiers) refers to *nirK* in AOB *Nitrosomonas* and NOB
 106 *Nitrospira*. *nirK/S*_(DEN) refers to *nirK/S* in denitrifiers only. *nirK/S*_(total) refers *nirK/S* in AOB,
 107 NOB, and denitrifiers. Values refer ratios between genes.

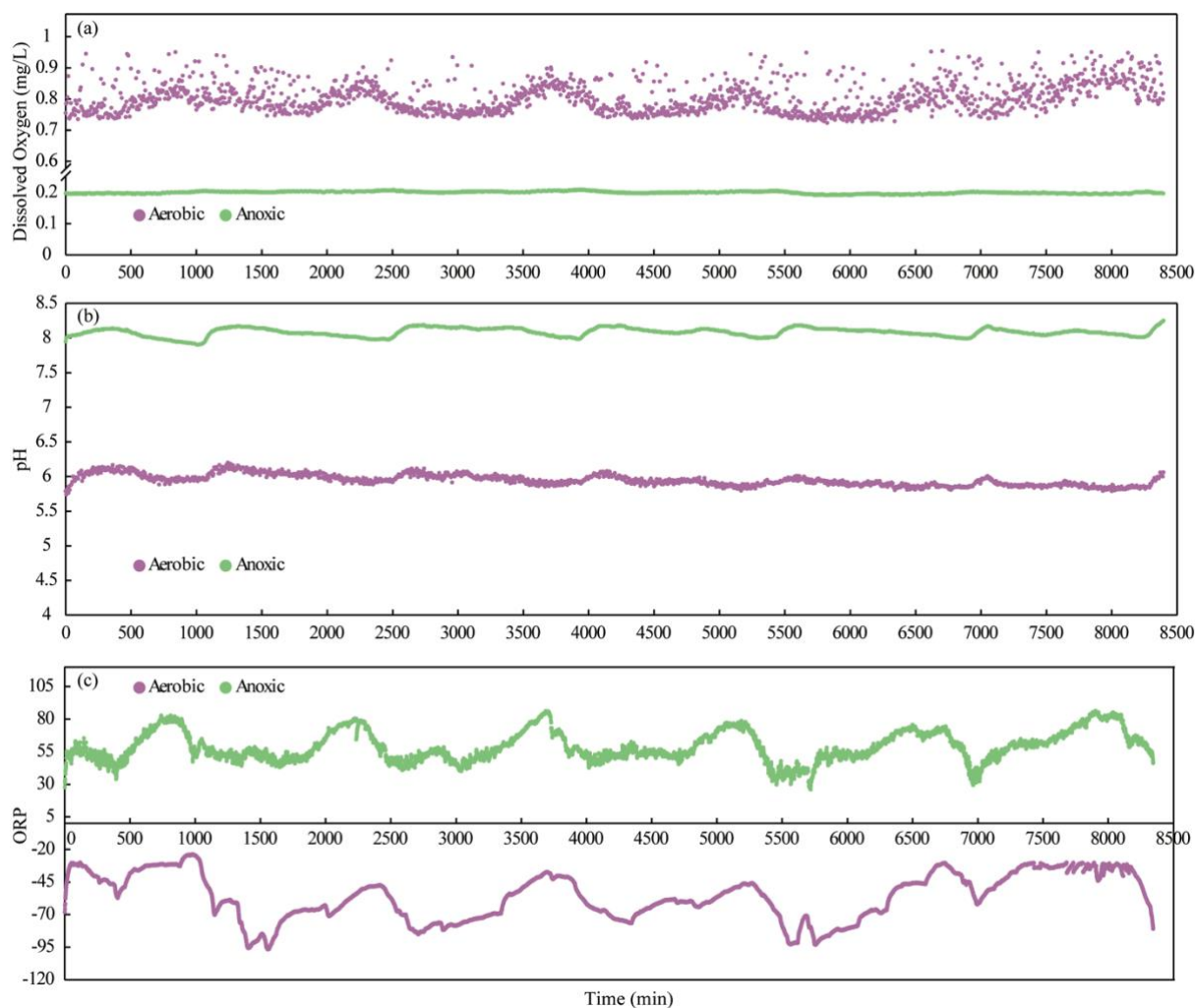


Fig. S8 Operational stability of dissolved oxygen, pH, and ORP in the Anoxic and Aerobic tanks during the study period. (a) Dissolved oxygen (DO). (b) pH. (c) Oxidation–reduction potential (ORP) monitored in the Anoxic and Aerobic tanks throughout operation. pH remained consistently higher in the Anoxic tank (green; ~7.9–8.2) than in the Aerobic tank (purple; ~5.7–6.1), consistent with alkalinity recovery in the Anoxic tank and alkalinity consumption in the Aerobic tank.

Electricity generation from produced CH₄ increased from 26274.6 kWh in Phase1 to 34858.0 kWh in Phase2.

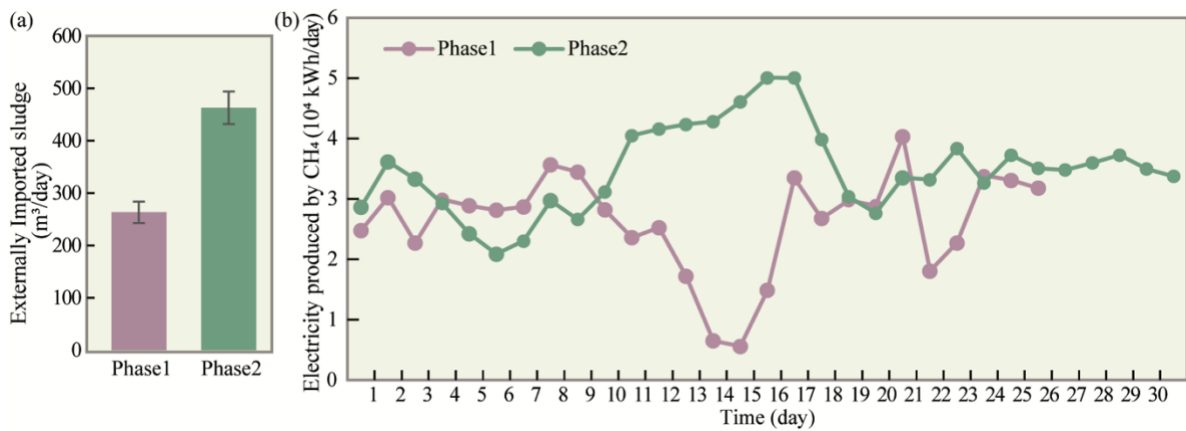


Fig. S9 Imported sludge loading and electricity generation from AD biogas-derived CH₄ during the two operational phases. (a) Volume of externally imported sludge for biogas recovery during the two sampling phases. (b) Monthly electricity generation from AD biogas-derived CH₄ during the two sampling phases. For each phase, 30 days of data are presented. Data averaged over one month were used for comparison between two phases.

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Table S1. UK Government GHG Conversion Factors and GHG offset using electricity produced from CH₄ across two phases.

Activity	Country	CO ₂ -eq.kg	CO ₂ -eq.kg of CO ₂ per unit	CO ₂ -eq.kg of CH ₄ per unit	CO ₂ -eq.kg of N ₂ O per unit	Unit
Electricity generated	Electricity: UK	0.17700	0.17489	0.00090	0.00122	kWh
Biogas	Biogas: UK	0.00022	-	-	-	kWh
	Electricity generation from AD biogas-derived CH ₄	CO ₂ -eq.kg produced – biogas	CO ₂ -eq.kg produced – electricity	Offset CO ₂ -eq.kg		
Phase1	26274.6 kWh	5.78	4650.6	1517.4		
Phase2	34858.0 kWh	7.67	6169.9			

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Table S2. Operation parameters for N₂O emissions calculation in side-stream and mainstream.

	Process	N removal percentage by pathway	TN removed	Flow rate	N ₂ O produced	N ₂ O produced in total	N ₂ O emission rate
		(%)	(mg-N/L)	(m ³ /day)	(g-N/(m ³ ·d) N ₂ O)	(g-N/(m ³ ·d) N ₂ O)	kg-N ₂ O/day
Phase1	PN/A	80.1	1810.5	650.0	5.84	9.59	6.23
	Denitrification	19.9			3.75		
Phase2	PN/A	42.6	742.0	668.1	1.31	5.86	3.81
	Denitrification	57.4			4.55		
Phase1	Mainstream	-	-	49727.0	-	-	-
Phase2	Mainstream	-	-	41132.1	-	-	-

Note: The percentage of nitrogen removal by each pathway in PN/A tank was predicted based on specific nitrogen removal activity results (Fig. S1).

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Table S3. Alpha diversity indexes.

Alpha diversity indices, including Chao1, (richness), Pielou (evenness), Shannon (combined richness and evenness), and Simpson (dominance), were used to characterize microbial community structure in each tank across the two phases. In Phase1, Chao1 index was 132 in the AD unit and 295 in the PN/A tank, both slightly reduced to 126 and 290, respectively, in Phase2, indicating a reduction in microbial richness in these tanks. Conversely, Chao1 increased from 520 and 501 in Phase1 to 563 and 544 in Phase2 in the Anoxic and Aerobic tanks, respectively, suggesting enhanced microbial richness in PN/A downstream tanks. The Pielou and Shannon indices slightly increased in the AD unit (Pielou: 1.06-fold, Shannon: 1.12-fold) but slightly decreased in the PN/A, Anoxic, and Aerobic tanks in Phase2 (ranging from 1.9 – 6.0% compared to Phase1). Meanwhile, the Simpson index exhibited marginal changes, with a 1.00- to 1.03-fold increase in Phase2. Overall, reduced evenness and diversity in AD unite downstream three tanks implies that some specialist taxa may have been favored in Phase2, leading to dominance of specific microbial groups and a corresponding decline in overall diversity.⁵

		AD unit				PN/A		Anoxic			Aerobic		139
Chao1	Phase1	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12
		133	144	120	299	280	307	478	556	527	503	475	526
	Phase2	S201	S202	S203	S204	S205	S206	S207	S208	S209	S210	S211	S212
		126	132	122	273	256	343	544	605	541	541	508	582
Simpson	Phase1	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12
		0.81	0.77	0.78	0.88	0.91	0.90	0.95	0.95	0.96	0.95	0.97	0.97
	Phase2	S201	S202	S203	S204	S205	S206	S207	S208	S209	S210	S211	S212
		0.81	0.73	0.81	0.87	0.91	0.87	0.93	0.94	0.92	0.93	0.94	0.95
Pielou	Phase1	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12
		0.45	0.45	0.44	0.50	0.54	0.52	0.63	0.61	0.66	0.64	0.70	0.67
	Phase2	S201	S202	S203	S204	S205	S206	S207	S208	S209	S210	S211	S212
		0.50	0.43	0.49	0.50	0.56	0.47	0.63	0.62	0.60	0.62	0.64	0.63
Shannon	Phase1	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12
		2.20	2.22	2.12	2.84	3.05	3.00	3.86	3.88	4.14	3.95	4.30	4.19
	Phase2	S201	S202	S203	S204	S205	S206	S207	S208	S209	S210	S211	S212
		2.41	2.10	2.35	2.82	3.11	2.77	3.95	3.97	3.79	3.92	3.97	3.98

Table S4. Genus-level assignment of methanogenesis-related genes in the AD unit.

	<i>Methanothrix</i>		<i>Methanobacterium</i>		<i>Methanoculleus</i>		<i>Methanosarcina</i>		Total	
	Pha1	Pha2	Pha1	Pha2	Pha1	Pha2	Pha1	Pha2	Pha1	Pha2
<i>ackA</i>	0.0	0.0	0.0	0.0	0.0	0.0	39.1	23.1	39.1	23.1
<i>pta</i>	0.0	0.0	0.0	0.0	0.0	0.0	39.6	23.7	39.6	23.7
<i>cdh</i>	0.3	0.8	17.3	11.5	4.0	2.9	199.1	120.5	275.9	169.4
<i>fwd</i>	0.0	0.4	143.8	89.8	29.2	28.1	785.3	481.8	1077.5	679.8
<i>ptr</i>	0.1	0.1	0.0	0.0	2.2	2.2	43.2	26.4	64.5	41.9
<i>mch</i>	0.0	0.0	19.5	11.6	1.9	1.9	41.7	24.0	63.1	37.6
<i>mta</i>	0.0	0.0	0.0	0.0	0.4	0.3	41.8	26.0	64.3	40.7
<i>mer</i>	0.0	0.1	18.0	10.8	0.4	0.3	40.3	24.9	60.7	38.1
<i>mtr</i>	0.0	0.7	151.0	96.5	18.3	21.7	86.0	50.8	276.9	183.0
<i>mcr</i>	0.1	0.6	123.6	74.5	8.3	8.5	314.2	193.9	506.2	317.9

141 Note: Pha1 and Pha2 refer to Phase1 and Phase2, respectively. Gene abundance is calculated
 142 as RPKM. Total represents total abundance of the gene in the community.

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Table S5 Size and operational parameters of each tank.

	PN/A	Anoxic	Aerobic
Depth, D_R (m)	3	3.5	3.5
Surface area (m ²)	300	530	5800
Volume, V_R (m ³)	900	1855	20300
pH	-	7.9~8.2	5.7~6.1
DO	-	0.19~0.21	0.72 ~ 1.02
ORP	-	-96.6~-23.4	26.2 ~ 86.5

Note: “-” represents that parameter was not routinely recorded onsite in the specific tank.

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Table S6 Phase classification

	Phase1	Phase2
	(Satisfied PN/A)	(Disturbed PN/A)
Period	Mar-2023	Aug-2023
TN of crude sewage (mg-N/L)	43.0	34.5
COD of crude sewage (mg/L)	524.2	549.0
Mainstream influent flow (m ³ /day)	41439	34277
TN of mainstream influent (mg-N/L)	37.3	38.9
PN/A influent flow (m ³ /day)	650.0	668.1
PN/A NH ₄ ⁺ (mg-N/L)	256.7	577.1
NH ₄ ⁺ volumetric loading (kg-N·m ⁻³ ·d ⁻¹)	1.5	1.0
NH ₄ ⁺ removal rate (kg-N·m ⁻³ ·d ⁻¹)	1.3	0.5
AD OLR (kg VS·m ⁻³ ·d ⁻¹)	~2.15	~3.35

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Table S7. Nitrogen and COD dosing for specific activity tests.

	Phase	Anammox	Denitrification	Nitrification
NH ₄ ⁺ -N	Phase1	120		240
(mg/L)	Phase2	240	-	480
NO ₂ ⁻ -N	Phase1	160	160	
(mg/L)	Phase2	300	300	-
COD	Phase1		300	
(mg/L)	Phase2	-	500	-

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Table S8. Sludge sample information for DNA extraction.

Sampling		Tank			
Phase	AD unit	PN/A	Anoxic	Aerobic	
Phase1	S01, S02, S03	S04, S05, S06	S07, S08, S09	S10, S11, S12	
Phase2	S201, S202, S203	S204, S205, S206	S207, S208, S209	S210, S211, S212	

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Table S9. On site information and constant value calculation for greenhouse gases emission calculation.

Onsite Parameters				Calculation Parameters		Constant Value	
	PN/A	Anoxic	Aerobic				
Depth, D_R (m)	3	3.5	3.5	$K_L \alpha_{N_2O, 20^\circ C}$ (/d)	81.87	$C_{N_2O, air}$ (g-N/m ³)	0.0003
Surface area (m ²)	300	530	5800				
Volume, V_R (m ³)	900	1855	20300	$K_L \alpha_{N_2O, process}$ (/d)	69.35	$K_L \alpha_{N_2O, T_{Process}}^{non-aerated}$ (/d)	2
Temp, $T_{Process}$ (°C)	33.0	13.0	13.0				
Total daily airflow, Q_A (m ³ /day)		1020000		$K_L \alpha_{O_2}$ (/h)	0.16	H_{N_2O}	0.025
Superficial gas velocity, v_g (m/s)		0.002035441					
Input power, P (W/m ³)		8.0		$K_L \alpha_{CH_4}$ (/h)	0.14		

167 **Supplementary Data file (Supplementary Data.xlsx) description**

168 Supplementary Data 1: Functional organisms' composition at genus level (relative
169 abundance %).

170 Supplementary Data 2: Abundance of nitrogen cycle related functional genes (RPKM).

171 Supplementary Data 3: Abundance of N₂O emission related functional genes in specific
172 bacteria in the Anoxic and Aerobic tanks.

173 Supplementary Data 4: Ratios between nitrite consuming enzyme *nirK/S* and N₂O consuming
174 enzyme *nosZ*.

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