

Supplementary Materials for

**Maifanite-enhanced pyrite biofilter for efficient autotrophic
denitrification of low-C/N mariculture tailwater: mechanistic insights
into microbial nitrogen–sulfur–iron coupling**

Jiannan Liu ^{a,b}, Mupindu Progress ^{a,b}, Xiangyu Yang ^{a,b}*, Yang-guo Zhao ^{a,b}*

*a Shandong Provincial Key Laboratory of Marine Environment and Geological Engineering (MEGE),
College of Environmental Science and Engineering, Ocean University of China, Qingdao 266100,
China*

*b Key Lab of Marine Environmental Science and Ecology (Ministry of Education), Ocean University of
China, Qingdao 266100, China*

**Corresponding authors:*

Phone: +86-0532-66786568 (Yang-guo Zhao); +86-0532-66782810 (Xiangyu Yang).

E-mail: ygzhao@ouc.edu.cn (Yang-guo Zhao); yangxiangyu@ouc.edu.cn (Xiangyu Yang).

Text S1. Optimization of pyrite-to-maifanite mixing ratio

This section describes the preliminary experiments conducted to determine the optimal mixing ratio of pyrite and maifanite for enhanced denitrification performance.

S1.1 Materials

Natural pyrite and maifanite were crushed and sieved to a particle size range of 3 ~ 5 mm, washed repeatedly with deionized water to remove surface powder, dried to constant weight at 105°C, and stored in a desiccator prior to use. The bulk densities of pyrite and maifanite were 2.7 and 1.3 g/cm³, respectively.

S1.2 Experimental design

Pyrite and maifanite were mixed at bulk volume ratios of 1:2, 1:1, 3:2, and 2:1, with pure pyrite as the control. All materials were pre-sieved to the same particle size range (3~5 mm) and measured by graduated cylinder under natural packing conditions without compaction. The total packing volume was kept consistent across all groups. Experiments were conducted in 250 mL serum bottles containing 100 mL of artificial seawater medium (initial NO₃⁻-N concentration: 50 mg/L, C/N = 0). Each bottle was inoculated with 10 mL of acclimated halotolerant denitrifying sludge (inoculum size: 10%, v/v). The initial pH was adjusted to 8.0 ± 0.1. After purging with N₂ for 15 min to remove dissolved oxygen, the bottles were immediately sealed and placed in a constant-temperature shaking incubator at 30°C and 120 r/min under dark conditions. All treatments were performed in triplicate.

S1.3 Analytical methods

After 48 h of incubation, liquid samples were collected from each bottle and filtered through 0.22 μm membrane filters. The concentrations of NO_3^- -N, NO_2^- -N, NH_4^+ -N, and total nitrogen (TN) were measured according to standard methods. TN removal efficiency was calculated as follows:

$$\text{TN removal efficiency (\%)} = \frac{\text{TN}_{\text{initial}} - \text{TN}_{\text{final}}}{\text{TN}_{\text{final}}} \times 100\% \quad (1)$$

S1.4 Results

The TN removal efficiencies at different mixing ratios are shown in Fig. S9. The pyrite–maifanite combination at a 1:1 (v/v) ratio achieved the highest TN removal efficiency ($99.18 \pm 5.93\%$), significantly outperforming the pyrite-only control ($54.05 \pm 1.33\%$) and other mixing ratios ($p < 0.05$). Further increasing the proportion of maifanite (1:2) did not yield additional improvement. The 1:1 ratio was therefore selected for the long-term biofilter experiments.

Text S2. Nitrogen mass balance estimation for inferring $\text{NH}_4^+\text{-N}$ removal pathway

To infer the fate of the removed $\text{NH}_4^+\text{-N}$, a simplified nitrogen mass balance was performed using the average concentrations of nitrogen species at Stage V.

Based on the data in Table S4, the nitrogen mass balance was calculated as follows:

- (1) Total nitrogen removed: $\Delta\text{TN} = 34.07 \pm 1.34 \text{ mg/L}$
- (2) Nitrogen removed via NO_3^- reduction: $\Delta\text{NO}_3^- = 23.67 \pm 1.01 \text{ mg/L}$
- (3) Unaccounted nitrogen loss = $\Delta\text{TN} - \Delta\text{NO}_3^- = 10.40 \pm 1.29 \text{ mg/L}$

The unaccounted nitrogen loss ($10.40 \pm 1.29 \text{ mg/L}$) was close to the measured $\text{NH}_4^+\text{-N}$ removal ($9.27 \pm 0.15 \text{ mg/L}$), with a minor discrepancy of 1.13 mg/L . This minor difference is likely attributable to analytical uncertainties and potential nitrogen incorporation into soluble microbial products or other minor sinks. Overall, the mass balance suggests that the removed $\text{NH}_4^+\text{-N}$ largely corresponded to the nitrogen fraction not explained by denitrification alone.

To further constrain the possible fate of $\text{NH}_4^+\text{-N}$, alternative removal pathways were systematically evaluated:

(1) Nitrification: The system was operated under oxygen-limited conditions ($\text{DO} < 1.0 \text{ mg/L}$), which inhibits the activity of $\text{NH}_4^+\text{-N}$ -oxidizing bacteria. Moreover, no net accumulation of NO_2^- or NO_3^- was observed in the effluent, ruling out significant nitrification.

(2) NH_3 volatilization: The reactor pH remained within the range of $7 \sim 8$ throughout the experiment. Under these conditions, free $\text{NH}_4^+\text{-N}$ concentration is negligible, and volatilization losses are considered minimal.

(3) Anaerobic ammonium oxidation (anammox): No known anammox-related genera (e.g., *Candidatus Brocadia*, *Candidatus Kuenenia*) were detected in the 16S rRNA gene sequencing analysis. Additionally, the stoichiometric requirements for anammox (simultaneous presence of NH_4^+ and NO_2^-) were not met under the operating conditions.

Given the exclusion of these alternative sinks, microbial assimilation remains the most plausible pathway for NH_4^+ -N removal. This inference is consistent with the increased microbial biomass and EPS production observed in the PM biofilter (see main text, Section 3.3). The assimilation of NH_4^+ -N into biomass is energetically favorable and commonly reported as the primary nitrogen sink in low C/N ratio systems ([Hou et al., 2022](#)).

It should be noted that this mass balance provides indirect evidence only. Direct confirmation of assimilatory NH_4^+ -N uptake would require ^{15}N isotope tracing or quantification of key assimilation gene *glnA*, which were beyond the scope of the present study and represent important directions for future work.

Text S3. Estimation of the relative contribution of autotrophic and heterotrophic denitrification

To estimate the relative contributions of autotrophic and heterotrophic denitrification in the PM biofilter, a simplified calculation was performed based on sulfate production stoichiometry at Stage V.

According to the stoichiometry of PAD process (Eq. 1 in main text), the reduction of 1 mg NO_3^- -N theoretically produces approximately 4.57 mg SO_4^{2-} (Fei et al., 2020). The average NO_3^- -N removal in the PM was 23.67 ± 1.01 mg/L at Stage V, and the net SO_4^{2-} production was 95.16 ± 21.23 mg/L, yielding an observed ratio of:

$$\text{Observed } \text{SO}_4^{2-} \text{ yield} = \frac{95.16}{23.67} = 4.02 \text{ mg } \text{SO}_4^{2-} \text{ per mg } \text{NO}_3^- - \text{N} \quad (2)$$

Assuming that all SO_4^{2-} production originated from sulfur-driven autotrophic denitrification, the fraction of NO_3^- -N removed via this pathway can be estimated as:

$$\begin{aligned} \text{Autotrophic contribution} &= \frac{\text{Observed } \text{SO}_4^{2-} \text{ yield}}{\text{Theoretical } \text{SO}_4^{2-} \text{ yield}} \times 100\% \\ &= \frac{4.02}{4.57} \times 100\% = 87.96\% \end{aligned} \quad (3)$$

The remaining fraction (~12.04%) is attributable to heterotrophic denitrification (supported by the observed TOC removal, Fig. 2c in main text) and iron-driven autotrophic denitrification via the NAFO pathway.

Several sources of uncertainty should be kept in mind when interpreting this estimate. Concurrent microbial sulfate reduction, which was detected in the PM through the enrichment of dissimilatory sulfate reduction genes (e.g., *dsrAB*, *sat*; Section 3.6.2), likely consumed a portion of the sulfate produced by autotrophic denitrification. If so, the actual autotrophic contribution would be higher than the 87.96% calculated above.

In addition, the iron-driven NAFO pathway does not generate sulfate as a byproduct, meaning any nitrate reduced via this route is not captured by a sulfate-based calculation. Intermediate sulfur species such as thiosulfate or elemental sulfur may also have formed transiently without being fully oxidized to sulfate. For these reasons, the 87.96% estimate should be viewed as a conservative approximation rather than a precise value.

That said, the broader conclusion that autotrophic denitrification dominated nitrate removal in the PM system does not rest on this calculation alone. At Stage V, the influent TOC was 29.97 ± 1.31 mg/L and the influent TN was 38.16 ± 0.76 mg/L (Table S3), yielding a C/N ratio of 0.79. With such a low C/N ratio, the available organic carbon is clearly insufficient to sustain heterotrophic denitrification as the primary nitrate removal pathway. The microbial community was overwhelmingly composed of known sulfur-oxidizing autotrophic denitrifiers such as *Sedimenticola*, *Thiobacillus*, and *Sulfurimonas*, while typical heterotrophic denitrifiers were far less abundant. Sulfur oxidation genes were enriched in the PM relative to the pyrite-only control, and sulfate was produced continuously throughout the 107-day experiment. Taken together, these independent observations converge on the same picture: autotrophic pathways, particularly sulfur-driven denitrification, carried the bulk of the nitrate removal.

A rigorous separation of autotrophic and heterotrophic contributions would require dedicated experiments such as ^{15}N isotope tracing or qPCR of key denitrification genes, which were beyond the scope of this study. This remains a meaningful direction for follow-up work.

Text S4. Microbial co-occurrence network analysis

To further elucidate the symbiotic and cooperative interactions among key functional genera underlying the enhanced denitrification performance in the PM biofilter, microbial co-occurrence network analyses were constructed for the PM and P biofilters at Stage V based on Pearson's coefficient > 0.8 and $p < 0.05$ by R programming language 4.4.2 and Gephi 0.11.2 (Wu et al., 2023). Only genera with relative abundance $> 0.1\%$ and at least one significant correlation were included in the network analysis.

Fig. S10 presents the co-occurrence networks for the PM (a) and P (b) biofilters, and Table S6 summarizes the topological parameters. The PM biofilter exhibited a higher number of nodes (66) than the P biofilter (59), indicating that maifanite addition supported a broader range of functionally active genera participating in network interactions. However, despite having more nodes, the PM network displayed lower average degree (5.36 vs. 7.36) and network density (0.083 vs. 0.127) compared to the P network. The PM network contained more nodes but fewer connections than the P network. This suggests that maifanite addition promoted a more modular and functionally specialized community structure, rather than simply increasing random or non-specific co-occurrences. In contrast, the higher density observed in the P network may reflect a more entangled and less differentiated interaction pattern, potentially including many weak or stress-induced correlations under suboptimal conditions (Berry and Widder, 2014).

Notably, in the PM network, a cluster of positive correlations was observed among key functional genera involved in nitrogen, sulfur, and iron metabolisms (Fig. S10a). *Sedimenticola* was positively correlated with *Sulfurimonas* (another sulfur-iron cycling genus) and *Marinobacter* (an EPS producer). *Marinobacter* also showed a positive correlation with *Hoeflea*, which is involved in iron metabolism. This interconnected positive network links sulfur-oxidizing autotrophic denitrifiers (*Sedimenticola*, *Sulfurimonas*), EPS-secreting bacteria (*Marinobacter*), and iron-cycling taxa (*Hoeflea*), suggesting a syntrophic consortium that coordinately drives the coupled nitrogen-sulfur-iron metabolism underlying the enhanced denitrification performance of the PM biofilter. In contrast, the P network lacked such tight cross-module connections, which may partially account for its inferior denitrification performance and higher NO_2^- -N accumulation.

In summary, the network analysis reveals that maifanite addition not only enriched functional guilds but also reprogrammed their ecological interactions, establishing a more syntrophic and functionally partitioned microbial consortium that underpins the efficient autotrophic denitrification observed in the PM biofilter.

Table S1. Heavy metals weight proportion (%) results of raw pyrite and maifanite, as determined by ICP-OES.

Element	Fe	Al	Zn	Mg	Mn	Cu	Mo
Pyrite	54.17	0.042	0.01	0.08	0.01	0	0
Maifanite	1.31	3.52	0.02	0.06	0.02	0.01	0.01

Table S2. Weight proportion (%) results of EDS before and after nitrogen removal.

Element	Before experiment	PM	P
C	13.28±1.34	9.55±1.35	11.68±2.56
O	9.39±0.76	39.01±2.35	5.01±1.52
P	0.27±0.07	0.86±0.08	0.78±0.16
S	38.55±3.01	24.03±1.27	37.79±3.82
Fe	38.50±1.67	26.55±1.35	44.74±2.34

Table S3. Nitrogen removal performance of different biofilters at various stages.**(a) Stage I**

Contaminant type	Biofilter type	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
NO ₃ ⁻ -N	PM	44.99±0.43	0.72±0.37 ^a	98.39±0.81 ^a
	P	44.99±0.43	0.74±0.53 ^a	98.36±1.17 ^a
NH ₄ ⁺ -N	PM	0	0.98±0.13 ^a	/
	P	0	0.95±0.34 ^a	/
NO ₂ ⁻ -N	PM	0	0.39±0.32 ^a	/
	P	0	2.81±0.41 ^b	/
TN	PM	44.99±0.43	2.09±0.65 ^a	95.35±1.46 ^a
	P	44.99±0.43	4.50±0.92 ^b	89.99±2.07 ^a

(b) Stage II

Contaminant type	Biofilter type	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
NO ₃ ⁻ -N	PM	45.24±0.69	7.53±0.69 ^a	83.09±1.40 ^b
	P	45.24±0.69	16.29±1.33 ^b	64.62±0.58 ^a
NH ₄ ⁺ -N	PM	0	0 ^a	100.00±0.00 ^a
	P	0	0 ^a	100.00±0.00 ^a
NO ₂ ⁻ -N	PM	0	0 ^a	/
	P	0	5.57±0.42 ^b	/
TN	PM	45.24±0.69	7.65±0.69 ^a	83.09±1.40 ^b
	P	45.24±0.69	20.67±0.44 ^b	54.30±1.07 ^a

(c) Stage III

Contaminant type	Biofilter type	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
NO ₃ ⁻ -N	PM	46.68±0.78	7.51±0.78 ^a	83.92±1.59 ^b
	P	46.68±0.78	16.56±1.16 ^b	64.52±2.35 ^a
NH ₄ ⁺ -N	PM	3.48±0.13	0 ^a	100.00±0.00 ^a
	P	3.48±0.13	0 ^a	100.00±0.00 ^a
NO ₂ ⁻ -N	PM	0	0 ^a	/
	P	0	5.97±0.57 ^b	/
TN	PM	50.16±0.82	7.51±0.78 ^a	85.03±1.51 ^b
	P	50.16±0.82	22.53±1.20 ^b	55.09±2.20 ^a

(d) Stage IV

Contaminant type	Biofilter type	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
NO ₃ ⁻ -N	PM	47.47±0.35	7.14±0.95 ^a	84.96±2.03 ^b
	P	47.47±0.35	21.91±1.31 ^b	53.84±3.57 ^a

NH ₄ ⁺ -N	PM	9.36±0.07	0 ^a	100.00±0.00 ^a
	P	9.36±0.07	0 ^a	100.00±0.00 ^a
NO ₂ ⁻ -N	PM	0	0 ^a	/
	P	0	5.29±0.08 ^b	/
TN	PM	56.83±0.30	7.14±0.95 ^a	87.44±1.69 ^b
	P	56.83±0.30	27.19±1.35 ^b	52.15±2.46 ^a

(e) Stage V

Contaminant type	Biofilter type	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
NO ₃ ⁻ -N	PM	27.76±0.68	4.09±0.48 ^a	85.24±1.95
	P	27.76±0.68	13.64±0.48 ^b	50.84±2.14
NH ₄ ⁺ -N	PM	9.27±0.15	0 ^a	100.00±0.00 ^a
	P	9.27±0.15	0 ^a	100.00±0.00 ^a
NO ₂ ⁻ -N	PM	0.13±0.02	0 ^a	100.00±0.00 ^a
	P	0.13±0.02	3.45±0.16 ^b	/
TN	PM	38.16±0.72	4.09±0.70 ^a	88.98±1.41 ^b
	P	38.16±0.72	17.09±0.41 ^b	53.99±1.44 ^a

$p < 0.05$.

Table S4. Microbial α -diversity indices of biofilms at Stage V.

Biofilter type	Good's coverage (%)	Chao1 index	Ace index	Shannon index	Simpson index
PM	99.39	1328.60	1394.27	5.59	0.93
P	99.54	1597.05	1668.53	5.37	0.92

Table S5. Influent and effluent nitrogen concentrations and calculated removal during at Stage V in the PM biofilter.

Contaminant type	Influent (mg/L)	Effluent (mg/L)	Removed (mg/L)
TN	38.16±0.72	4.09±0.70	34.07±1.34
NO ₃ ⁻ -N	27.76±0.68	4.09±0.48	23.67±1.01
NH ₄ ⁺ -N	9.27±0.15	0	9.27±0.15
NO ₂ ⁻ -N	0	0	0

Table S6. Topological parameters of microbial co-occurrence networks in the PM and P.

Parameter	PM	P
Nodes	66	59
Edges	177	217
Average degree	5.36	7.36
Network density	0.083	0.127

Note: Networks were constructed based on Pearson's coefficient > 0.8 and $p < 0.05$.

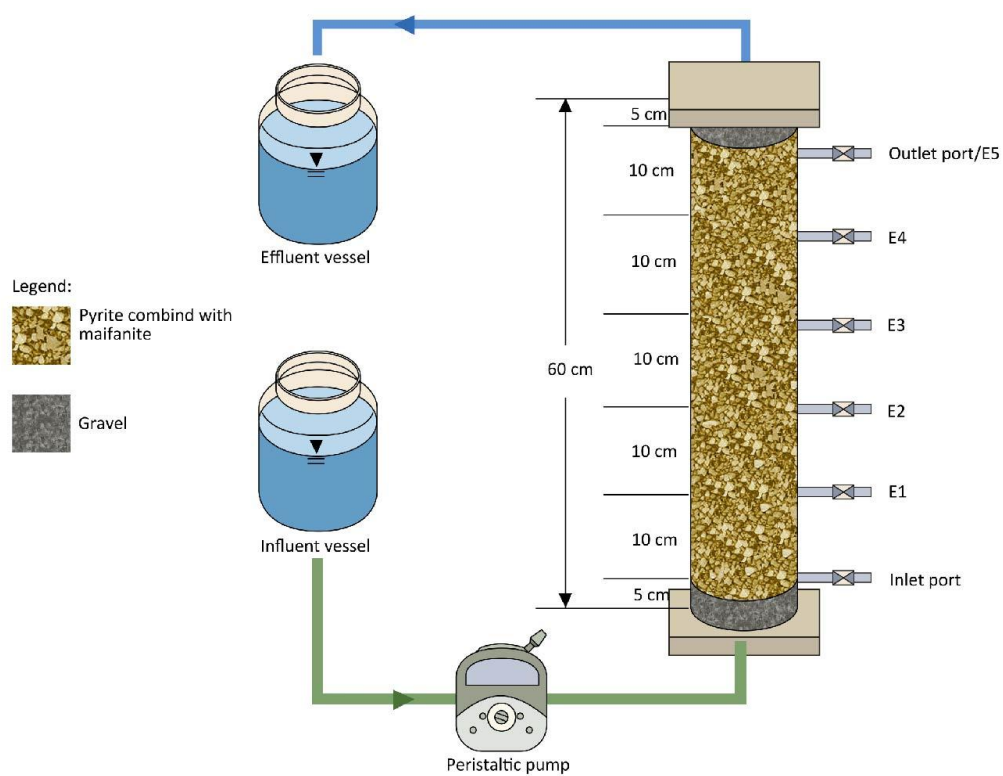


Fig. S1. Schematic diagram of pyrite-driven biofilters

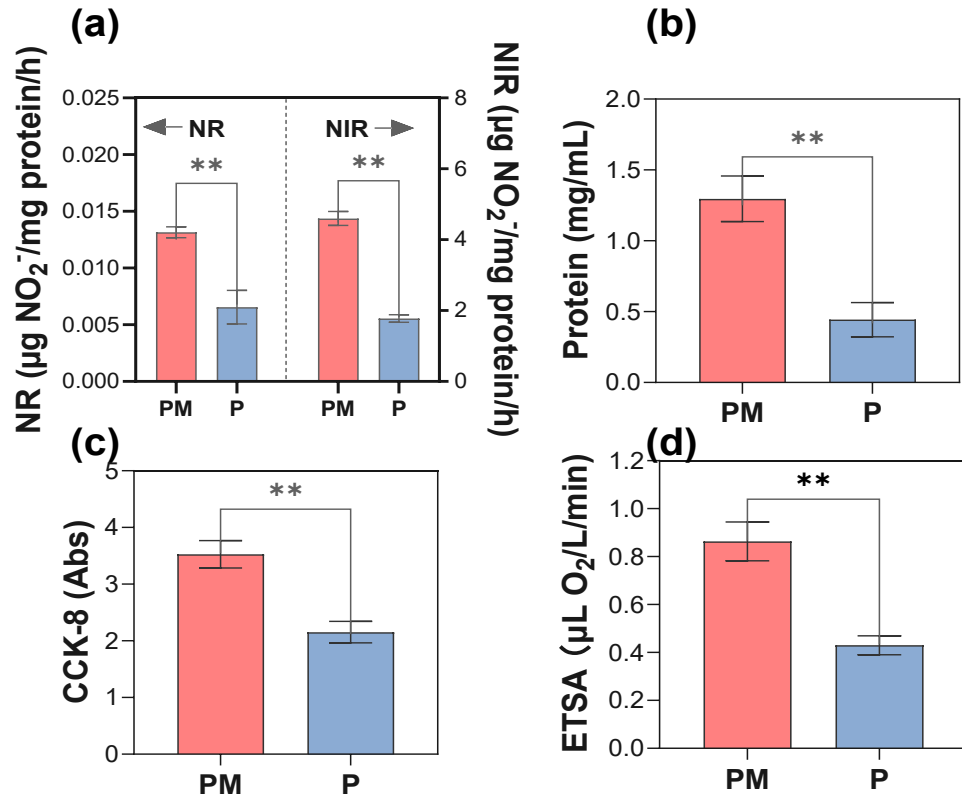


Fig. S2. Variations of (a) NR and NIR enzymes; (b) Total protein concentration; (c) Relative number of live cells of biofilms; and (d) Electron transport activity at Stage V.

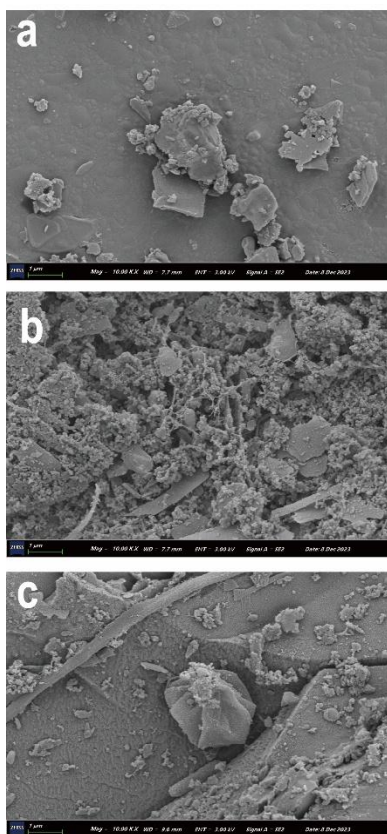


Fig. S3. SEM images of carriers (a) before operation, and after operation: (b) PM and (c) P.

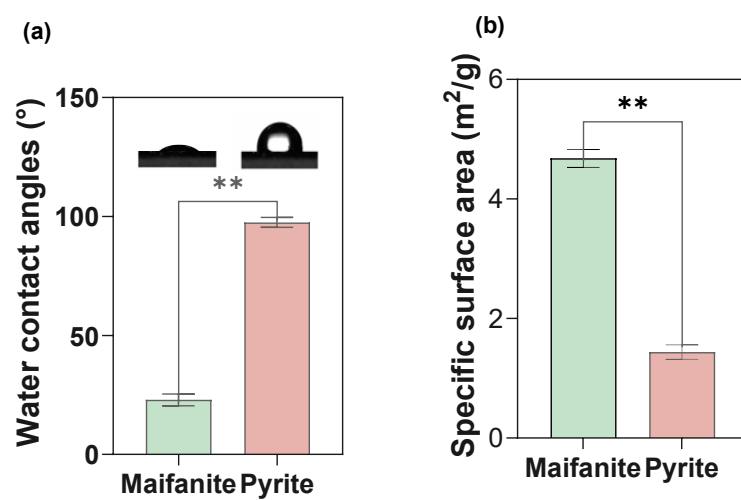


Fig. S4. (a) Surface WCA, and (b) Specific surface area of maifanite and pyrite.

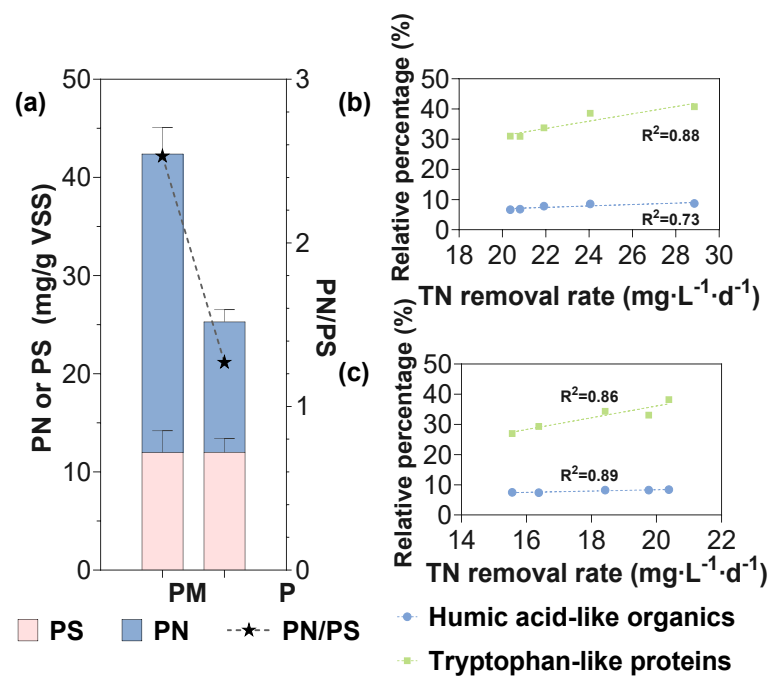


Fig. S5. (a) Variations in the PN and PS contents of EPS at Stage V; Correlations between the TN removal rate and the relative percentage of tryptophan-like and humic acid-like organics in the (b) PM and (c) P.

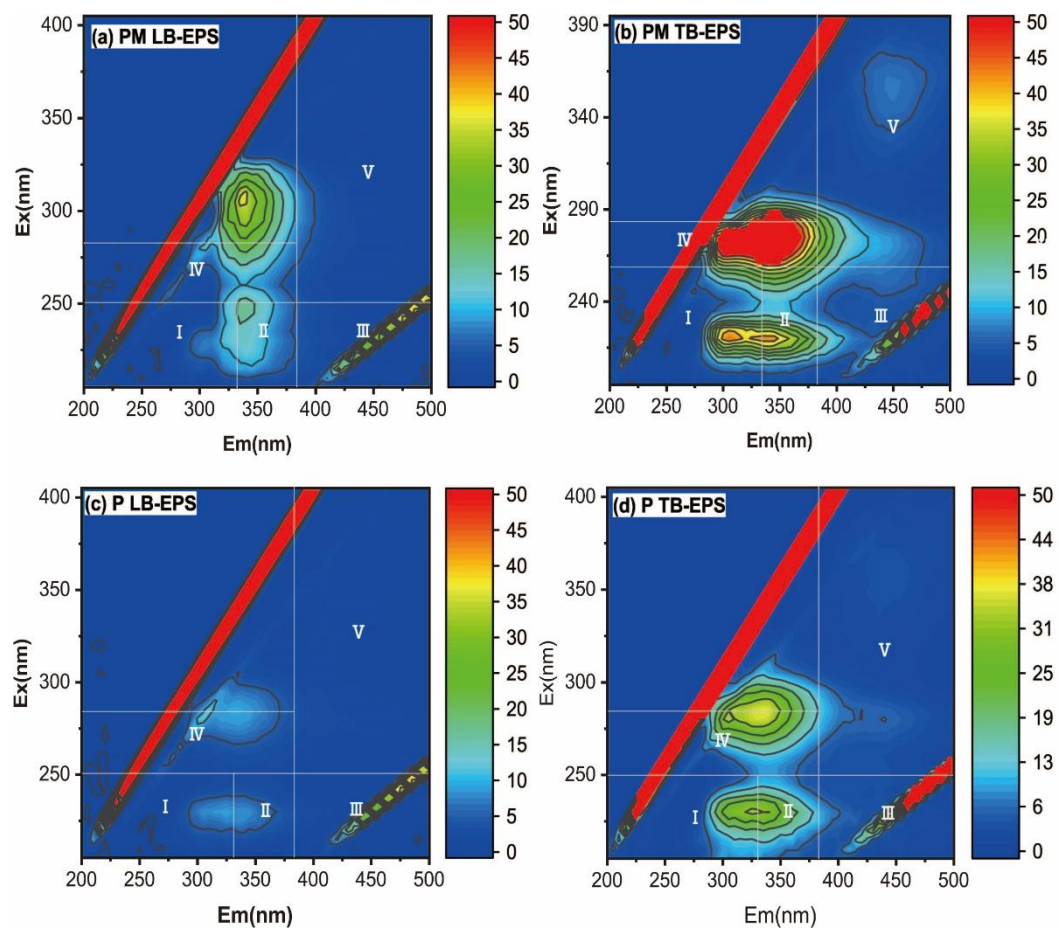


Fig. S6. 3D-EEM Fluorescence spectra of EPS in different biofilters at Stage V. (a. PM LB-EPS; b. PM TB-EPS; c. P LB-EPS; d. P TB-EPS.)

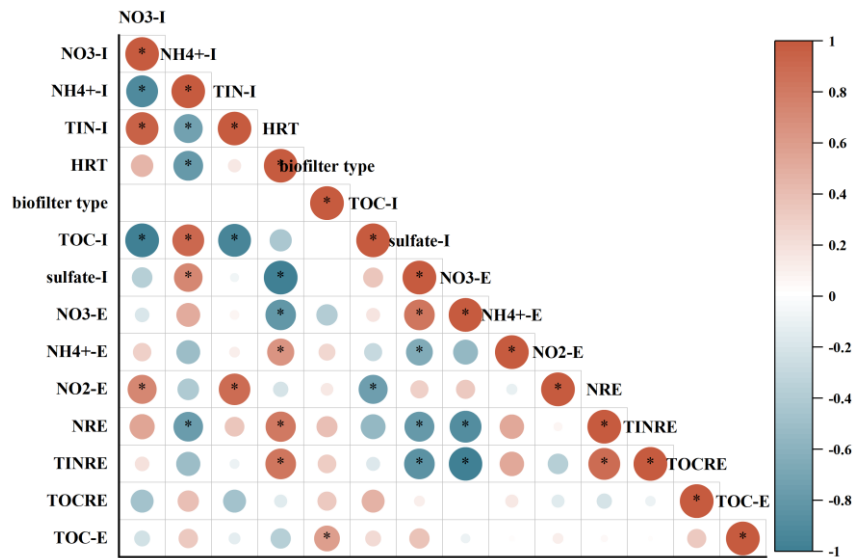


Fig. S7. The correlation analysis between nitrogen treatment performances and operation parameters. (A * was marked as $p < 0.05$.)

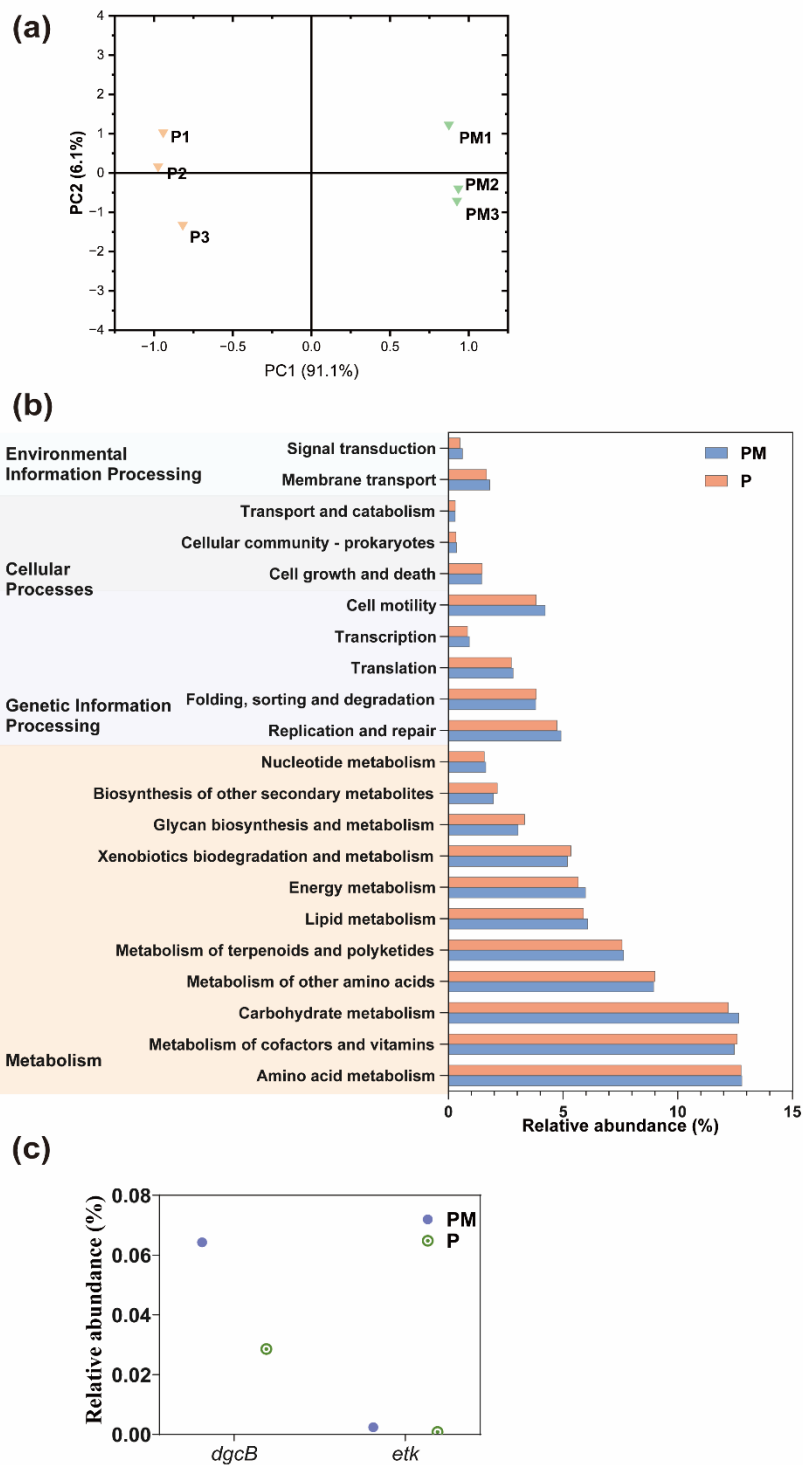


Fig. S8. Microbial compositions at Stage V (a) PCoA analysis; (b) The predicted microbial functions based on KEGG annotation; (c) The abundance of functional genes associated with EPS production.

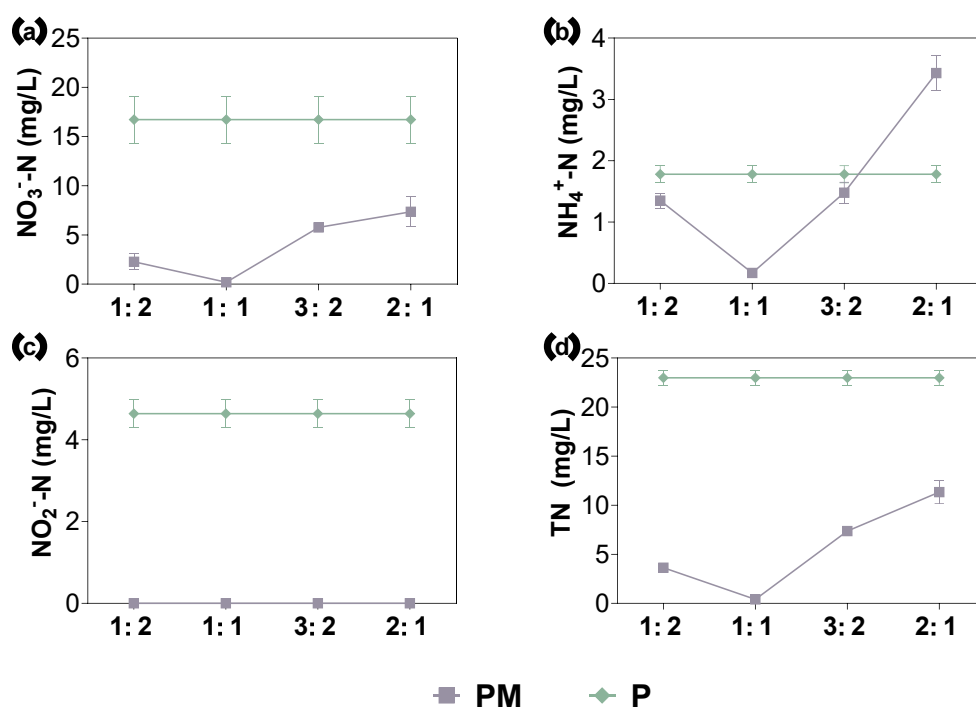


Fig. S9. Effects of different dosages on various pyrite-driven denitrification enhancement systems: (a) effluent NO_3^- -N; (b) effluent NH_4^+ -N; (c) effluent NO_2^- -N; and (d) effluent TN.

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