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Combined application of nitrate and schwertmannite promotes As(III) immobilization and greenhouse gas emission reduction in flooded paddy fields

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ABSTRACT

The challenge of concurrently mitigating arsenic (As) mobilization and greenhouse gas (GHG) emissions presents a significant obstacle in paddy fields, as single-factor approaches often create trade-offs. Our study aims to overcome this by evaluating a novel nitrate and schwertmannite (nitrate+Sch) co-amendment. This study demonstrated the efficacy of nitrate+Sch co-amendments for simultaneous suppression of As(III) mobilization and GHG emissions in flooded paddy fields. Following a 10-day incubation, the combined amendment outperformed the nitrate-alone treatment by completely immobilizing the initial 0.1 mM dissolved As(III) (final $0.11 \pm 0.02 \mu\text{M}$; $n = 3$) and reducing N_2O , CH_4 , and CO_2 emissions by 46.8 %, 26.3 %, and 7.4 %, respectively. The synergistic effect is attributed to the coupling of multiple biogeochemical processes. Attenuated As(III) mobilization was ascribed to adsorption by Fe(III)-oxides/oxyhydroxides regenerated from successive cycles of Feammox and nitrate-reducing coupled with iron oxidation (NRFO), which regenerated Fe(III) oxides for As adsorption and promoted complete denitrification to N_2 . As NRFO has a high thermodynamic feasibility, it promoted full denitrification to N_2 rather than N_2O . Moreover, the combined treatment created multiple electron acceptors for the anaerobic oxidation of methane (AOM) network linked to reduction of NO_3^- , Fe(III) and SO_4^{2-} , thereby leading to anaerobic oxidation of CH_4 to CO_2 . A portion of the resulting CO_2 might be sequestered into poorly-soluble carbonate minerals leading to lower CO_2 emission to the atmosphere. Metagenomic sequencing revealed that nitrate+Sch amendment enriched the abundances of key microorganisms linked to As/Fe oxidation and GHG mitigation (e.g., *Streptomyces*, *Pseudomonas*, *Candidatus Methanoperedens*, *Thermomonas*, and *Desulfosarcina*). Our findings document the efficacy of nitrate+Sch amendments as an effective remediation strategy to simultaneously address As(III) pollution and GHG emissions in flooded paddy fields. This approach offers a promising pathway towards more sustainable rice cultivation by addressing two critical environmental issues simultaneously.

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1. Introduction

Flooded paddy fields are significant anthropogenic sources of arsenic (As) pollution and greenhouse gas (GHG) emissions, posing serious threats to environmental quality and food security [1,2]. Under anoxic conditions, the reductive dissolution of As(V)/Fe(III)-bearing minerals, mediated by dissimilatory metal-reducing bacteria, mobilizes the more toxic and bioavailable As(III) species [3]. Concurrently, these anoxic conditions facilitate the microbial production of potent GHGs, notably methane (CH₄) and nitrous oxide (N₂O) [4]. Microbial remediation strategies offer a promising approach to mitigate these issues [5]. However, a significant challenge arises from the inherent conflicts between the biogeochemical pathways involved. For instance, anaerobic methane oxidation coupled with As(V) reduction (AOM-AsR) as mediated by AOM bacteria reduces CH₄ emission but concurrently promotes As(III) mobilization [6]. Likewise, microbial nitrate-reducing As(III) oxidation (NRAO) facilitates As(III) immobilization but often leads to increased N₂O release due to incomplete denitrification [7]. These antagonistic microbial processes highlight a critical research gap: the lack of a synergistic strategy that can simultaneously achieve As(III) immobilization and reduction of both CH₄ and N₂O emissions in flooded paddy soils.

Given the inherent conflict between As(III) immobilization and GHG mitigation (e.g., the competition between the AOM-AsR and NRAO pathways), alternative remediation strategies are urgently needed [8]. In natural systems, microorganisms and minerals often exhibit co-evolved relationships that enable thermodynamically favorable interactions within microbe-mineral associations [9]. Specifically, FeO_x minerals possess high surface area and oxidation potential, allowing efficient oxidation and adsorption of As(III) to facilitate its immobilization [8,10]. The addition of such minerals can also promote the oxidation of CH₄ to CO₂ via metal-driven anaerobic oxidation of methane (AOM) [11], while suppressing N₂O production by inhibiting denitrifying enzymes [12]. These effects suggest that the input of selected minerals can redirect microbial processes to simultaneously achieve As(III) immobilization and GHG reduction in flooded paddy soils. However, given the opposing electron flow requirements between As(III)/CH₄ oxidation and N₂O reduction, clarifying the mechanisms of electron transfer synergy and competition in microbe-mineral systems is essential for reconciling these conflicting pathways.

To address this challenge, we propose a novel strategy centered on the application of schwertmannite (Sch, Fe₈O₈(OH)_{8-2x}(SO₄)_x), a metastable iron oxyhydroxysulfate mineral. Schwertmannite is a compelling amendment due to its high specific surface area and strong affinity for adsorbing As [13]. More importantly, its structure contains multiple potential electron acceptors (Fe(III) and SO₄²⁻), suggesting the potential to regulate key microbial processes. We hypothesize that Sch amendment can: (i) directly immobilize As(III) through adsorption and co-precipitation; (ii) mitigate CH₄ emissions by serving as an electron acceptor for anaerobic methane oxidation coupled to Fe(III) or sulfate reduction (AOM-FeR/SAOM) [14,15]; and (iii) suppress N₂O emissions by facilitating the complete denitrification of nitrate via Fe(II)-driven nitrate reduction, a thermodynamically favorable process (see Table S1).

Despite the individual promise of these mechanisms, the integrated biogeochemical interactions between Sch and the native microbial community in a complex system like a paddy soil, particularly in the presence of nitrate, remain poorly understood. The potential synergistic or antagonistic effects on the intertwined Fe, As, C, N, and S cycles are unknown. Therefore, the novelty of this work lies in systematically investigating the simultaneous effects of Sch and nitrate on the co-optimization of As sequestration and GHG mitigation.

This study aims to elucidate the mechanisms by which combined Sch and nitrate amendment influences As immobilization, denitrification, and methane oxidation in flooded paddy soils. Through a combination of mineralogical analyses, enzymatic activity assays, and metagenomic

sequencing, we seek to profile the associated microbial pathways and redox processes. The ultimate goal is to construct a conceptual model of the coupled biogeochemical cycles, providing a scientific basis for developing effective and scalable remediation strategies for contaminated paddy fields.

2. Materials and methods

2.1. Collection and properties of As-enriched paddy soils

As-contaminated soil was collected from a paddy field receiving As inputs from realgar mine tailings located in Shimen County (29°58' N, 111°38' E) of Changde City, Hunan Province, China. During rice peak tillering stage, multiple soil cores (25 × 25 × 10 cm³) were collected from a depth of 10–30 cm using a soil corer, and then homogenized to form a composite sample. The composite sample was sealed in sterile plastic bags and transported to the lab. To maintain the indigenous bacteria, fresh soil was sealed in polyethylene vinyl containers and stored at 4 °C in the dark until used. Prior to experimentation, subsamples were air-dried at room temperature and sieved through a 2-mm mesh for soil characterization (Method S1). The paddy soil used in the experimental trials was an acidic red clay (pH = 5.8) with high concentrations of total arsenic (80.2 ± 7.1 mg/kg) and NH₄⁺-N (0.54 ± 0.10 g/kg) in background. Additional physicochemical parameters of the paddy soil are listed in Table S2.

2.2. Synthesis and characterization of schwertmannite

Synthesis of Sch was conducted according to the protocol described by Regenspurg et al. [16]. In brief, 38.92 g FeSO₄·7 H₂O was completely dissolved in 1750 mL Milli-Q water and slowly mixed with 21 mL 30 % H₂O₂ through pipette injection. The resulting suspension was stirred at 500 rpm with a magnetic stirrer for 24 h at room temperature. Precipitates were separated by centrifugation at 5000 rpm followed by washing with Milli-Q water until the pH reached a constant value (pH ~ 4.0). Finally, the brown Sch particles were harvested by freeze-drying and stored in a vacuum drying chamber in the dark. X-ray diffraction (XRD) and transmission electron microscopy (TEM) were employed to confirm the properties of the synthesized Sch (Fig. S1).

2.3. Experimental design

Soil-water (mass/volume ratio of 1:3) microcosms were employed to simulate flooded-anoxic, As-contaminated paddy fields. Anoxic microcosms were performed in 50-mL sealed serum bottles. Following the experimental design of previous investigations [11], dissolved As(III) was directly added into microcosms to evaluate As(III) immobilization behavior under simulated flooded As(III)-rich soil conditions. Briefly, each microcosm was comprised of 10 g fresh paddy soil (<2 mm), 30 mL reaction solution (0.1 mM As(III) and 0.2 mM acetate with/without 20 mM nitrate). Prior to incubation, all reaction solutions and Sch particles were autoclaved for 20 min at 120 °C to inactivate foreign microbes. The experimental treatments were comprised of a control (CK), nitrate-alone, Sch-alone and nitrate+Sch. Supplemented dosages of Sch were set as 0.06 and 0.10 g. All treatments were conducted in triplicate, as summarized in Table S3. Each incubation bottle represented an independent biological replicate derived from distinct soil subsamples.

To mimic flooded-anoxic conditions, assays were deoxygenated by purging with pure N₂ for 60 min (liquid phase for 30 min followed by head space for 30 min) and sealed with butyl rubber stoppers and aluminum caps. Microcosms were cultured at 30 °C in the dark for a 10-d incubation period. The suspensions, headspace gases and soils derived from the incubations on days 2, 4, 6, 8 and 10 were collected to evaluate As(III) mobilization/immobilization and GHG emissions.

To examine changes in As, Fe, C, O and S speciation due to interactions between Sch and microbes, 0.10 g Sch and 2.0 mL of the

reaction solution (containing 2.0 mM acetate, 0.10 mM As(III) and 20 mM nitrate) were sealed in a semi-permeable dialysis bag with a molecular weight cutoff of 10000 Daltons. The dialysis bag allowed the exchange of released ions and electrons, while preventing direct contact between the Sch and soil particles. The sealed dialysis bags were placed on the soil surface and incubated in the aforementioned microcosms (10 g fresh soil + 28 mL reaction solution). After incubating for 8 days, Sch particles in the dialysis bag were isolated by freeze-drying for characterization of morphology and As/Fe/C/O/S species.

2.4. Analytical methods

2.4.1. Determination of As and Fe species

Concentrations of dissolved As and Fe in the flooded layer were determined to examine (im)mobilization of As in the flooded paddy fields. Prior to determination, the supernatant was extracted and filtered through sterile 0.22 μm filters (nylon, Jinteng, China). Dissolved Fe(II) and Fe(T) (total Fe) were measured immediately using the phenanthroline method with a UV-VIS spectrophotometer (UV-2802, UNICO, China) [17]. The Fe(III) concentration was determined as the difference between Fe(T) and Fe(II). Concentrations of As(III) and As(V) were determined by HPLC coupled to an atomic fluorescence spectrometer (HPLC-AFS, SA-20, Jitian, Beijing, China) [18], while the concentration of As(T) (total arsenic) was determined by ICP-MS (NexION 2000, PerkinElmer, USA) [19].

2.4.2. Determination of C, N and S species

N conversion of the current study was strategically designed to build directly upon our group's prior [11]. Concentrations of nitrate (NO_3^-), nitrite (NO_2^-), nitrous oxide (N_2O) and soil-derived ammonium (NH_4^+) were determined to assess denitrification and anaerobic oxidation of ammonia (anammox and Feammox) across the different treatments. Soil derived $\text{NH}_4^+\text{-N}$ was collected by KCl (1 M) extraction [20]. Concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ were quantified using the salicylate, hydrazine reduction and sulfanilamide/N-1-naphthylethylene spectroscopy methods with a continuous flow analyzer (Autoanalyser-3, Seal, Germany) [21]. Dissolved organic carbon (DOC) in the flooded layer was quantified using a TOC analyzer (Shimadzu, TOC-L CPH, Japan) to examine the loss of DOC linked to CH_4 and CO_2 generation [22]. The 4-amino-N, N-dimethyl aniline spectrophotometry method was employed to determine sulfide (S^{2-}) concentrations resulting from reduction of Sch-derived SO_4^{2-} involved in SAOM and/or As(V)/Fe(II) reduction processes [23]. Head-space gas samples were extracted with a syringe and injected into 25-mL vacuum bottles. Concentrations of CH_4 , CO_2 and N_2O were measured using a gas chromatograph (GC, Agilent Technologies 7890B, USA) equipped with a TCD to measure CH_4 and CO_2 and an ECD and Poropak Q (80/100) column to detect N_2O [24]. All chemical reagents utilized in the present work were of superior grade.

2.4.3. Characterizations of minerals

TEM-mapping was employed to characterize the morphology and distributions of As/Fe/O/C/S adsorbed on the surfaces of Sch particles that were sealed in dialysis bags. Speciation of adsorbed As/Fe/O/C/S was assessed by XPS (ESCALAB 250Xi, Thermo, USA). A full range spectrum incorporating As3d, Fe2p, O1s, C1s and S2p was scanned and the related energy spectra were profiled using Avantage software 6.6.0.

2.4.4. Determination of denitrifying enzymes and Mcr

The production of N_2O and CH_4 is closely influenced by the activities of key enzymes responsible for denitrification and methanogenesis/methanotrophy [19,25]. Therein, the activities of denitrifying enzymes (NAR [$\text{NO}_3^- \rightarrow \text{NO}_2^-$], NIR [$\text{NO}_2^- \rightarrow \text{NO}$], NOR [$\text{NO} \rightarrow \text{N}_2\text{O}$] and NOS [$\text{N}_2\text{O} \rightarrow \text{N}_2$]) [11] were assessed. Similarly, the Mcr-mediated enzyme catalytic process serves as a key enzyme regulating the turnover of CH_4 production/oxidation by methanogens and methanotrophs in anoxic-flooded soils [26]. Hence, the activities of NAR, NIR, NOR, NOS

and Mcr were determined using an ELISA Kit (Jiangsu Meimian Industrial, China) to assess the impacts of the treatments of nitrate with/without Sch on the activities of key enzymes influencing the generation of N_2O and CH_4 [27]. Details of the commercial assay kits used to determine the activities of the target enzymes are presented in Table S4. All standard curves for the enzyme assays are provided in Fig. S2.

2.5. DNA extraction, library construction, and metagenomic sequencing

After a 10-d incubation, soil samples derived from CK, nitrate, 0.10-g Sch, and nitrate+ 0.10-g Sch groups were collected for metagenomic sequencing. Total genomic DNA was extracted from 0.5-g soil using the Mag-Bind® Soil DNA kit (Omega Bio-tek, USA) following manufacturer's instructions. The DNA extracted from the replicate samples ($n = 3$) of each group was mixed to form a composite sample for sequencing. The concentration and purity of extracted DNA were determined using a NanoDrop2000. The quality and integrity of DNA extracts were assessed on 1 % agarose gel. DNA extract was fragmented to an average size of about 400 bp using Covaris M220 (Gene Company Limited, China) for paired-end library construction, which was constructed using NEXTFLEX® Rapid DNA-Seq (Bioo Scientific, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt end of fragments. Paired-end sequencing was performed on an Illumina NovaSeq (Illumina Inc., USA) at Majorbio Bio-Pharm Technology (Shanghai, China) using NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles) according to manufacturer's instructions (www.illumina.com).

Sequencing libraries were established with a NEBNext®Ultra™ DNA Library Prep Kit for Illumina® (NEB, USA). AgaroAgencourt AMPure XP (Beckman, USA) was used for product purification. The GenNext™ NGS Library Quantification Kit (Toyobo, Japan) was used for gene prediction and Salmon (Version 1.3) was used for gene quantification [19]. The libraries were sequenced using the Illumina Novaseq 6000, 150-bp paired-end technology at TinyGen BioTech (Shanghai). The metabolic pathways of bacteria were predicted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [28]. Detailed information regarding the sequencing primers and protocols is provided in Method S2. The metagenomic sequence data sets were submitted to NCBI Sequence Read Archive.

2.6. Data analysis

Data were analyzed and visualized using Origin 2024b, Microsoft Excel 2017, SPSS 27.0, and packages (ggplot2, vegan, linkET and dplyr) of R 4.4.2. All values are presented as the mean $\pm$ standard deviation (SD) of triplicate analyses. Treatment differences were assessed by analysis of variance using SPSS with a 95 % confidence level ($P < 0.05$).

3. Results and discussion

3.1. Changes in Fe and As concentrations and speciation of flooded paddy soils

Throughout the 10-d incubation period, the maximum Fe(II) concentrations were up to 0.19 and 0.24 mM on the 8th day for the 0.06 and 0.10 g Sch amendments, which were nearly 10 and 13 times that of the CK group, respectively (Fig. 1b). The phenomenon was ascribed to Sch is a poorly-crystalline Fe(III)-oxyhydroxysulfate mineral that can be reduced by Feammox bacteria and dissimilatory metal reduction bacteria (DMRB) [29,30]. By contrast, nitrate addition led to an opposing pattern for dissimilatory Fe(III) reduction as virtually no soluble Fe(T) was detectable in assays amended with nitrate alone or nitrate+Sch mixtures (Fig. 1a). The suppressed Fe(III) reductive dissolution was mainly ascribed to nitrate fostering an enhanced NRFO performance [7].

Although the additions of Sch led to a large decline of As(III) during the initial 2 days of the incubation due to adsorption by Sch, the As(III)

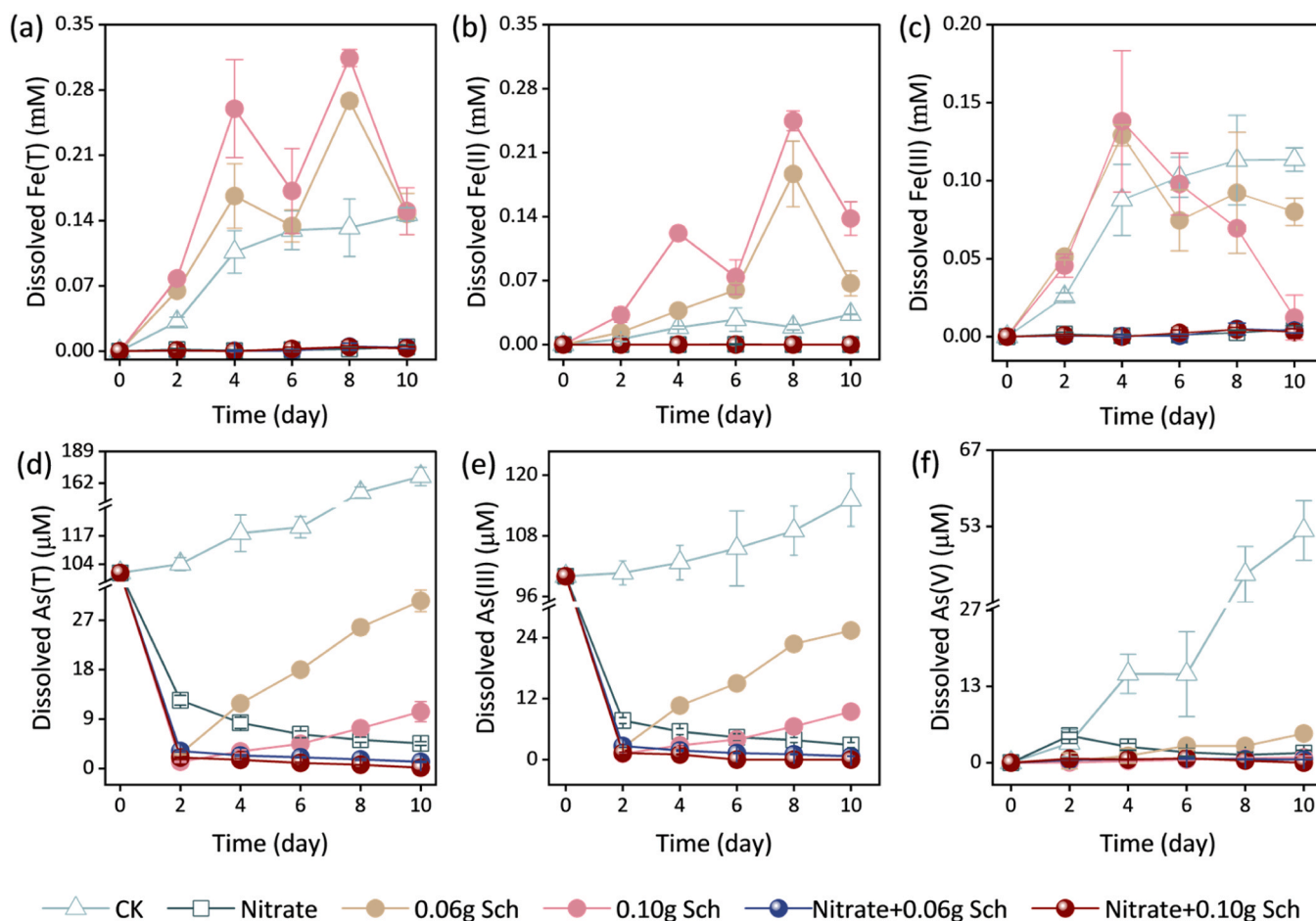


Fig. 1. Variations Fe and As species concentrations in the flooded layer of paddy soil microcosms during a 10-d incubation ($n = 3$). Subgraphs a-c indicate Fe(T), Fe(II), and Fe(III) concentrations, whereas the subgraphs d-e indicate As(T), As(III) and As(V) concentrations. The error bars represent SD of three replicates.

concentration gradually increased thereafter and reached peak values (nearly 25.4 and 9.4 μM for 0.06 and 0.10 g Sch) on the 10th day. The release of As(III) was attributed to Fe(III) reduction from Sch accompanied by the reductive dissolution of As(V) [30]. The poor As(III) immobilization performance implies that the Sch treatment provided only limited remediation capacity for addressing As(III) pollution in flooded paddy soils. Conversely, amendment with nitrate-alone triggered a stronger As(III) immobilization effect than Sch-alone, as determined by the removal of As(III) (>91 %) and production of As(V) (1.70 μM) at the end of the incubation period (Fig. 1e-f). However, the combined treatment of nitrate+Sch enhanced the immobilization of As(III). Since As(III) is primarily immobilized via DRFO or oxidized via NRAO [6], the coexistence of nitrate+Sch fostered a reliable As(III) immobilization performance as virtually no As(T) was released during the incubation period (Fig. 1d). Given the low dissolved As concentrations susceptible to leaching on the 10th day and contributing to environmental risk (Fig. S4), the combined nitrate+Sch treatment provides the best efficacy (removal rates >97 %) for remediating As(III) pollution in flooded paddy soils.

3.2. N redox transformations linked to N_2O emission

Since nitrate is an essential component for sustaining reliable As(III) immobilization (Fig. 1), we next examined GHG emissions and enzyme activities with a focus on the nitrate-supplemented assays. The residual nitrate and nitrite concentrations from the different treatments followed nitrate+ 0.10 g Sch < nitrate+ 0.06 g Sch < nitrate-alone (Fig. 2b-c). This pattern indicates that the Sch treatments promote the consumption

of nitrate and nitrite. The greater loss of nitrate and nitrite from the nitrate+Sch combined treatments may result from nitrate and nitrite participating in the oxidation of Fe(II). Specifically, the competitive reduction of exogenous Sch and Fe(II) oxidation through NRFO prevents the dissolution and leaching of sediment-derived Fe(T) (Fig. 1a) [7]. Theoretically, the loss of NO_3^- -N amount should be higher than the sum of residual NO_2^- -N and N_2O -N amounts according to the N mass balance associated with denitrification. Conversely, the loss of NO_3^- -N was less than the sum of NO_2^- -N and N_2O -N in the nitrate+Sch combined treatments (Fig. 2b-d). We attribute the lower amount of nitrate lost than the assumed nitrate consumption via denitrification to the combined nitrate+Sch treatments intensifying the consumption of ammonia (Fig. S5) and producing nitrate via the Feammox pathway (Eq. 2 of Table S1).

With respect to N_2O emissions, the nitrate+Sch combined treatments (0.06 and 0.10 g) induced a remarkable suppression of N_2O emission, as N_2O emissions declined by 39.8–46.8 % versus the nitrate-alone treatment on the 10th day. This attenuation in N_2O emission might be ascribed to NRFO competitively outperforming NRAO (-483.0 vs. -8.7 KJ/mol in Gibbs free energy; Table S1) and the high production of Fe(II) resulting from Sch reduction (Fig. 1b) that thermodynamically favors full denitrification by NRFO. Hence, the coexistence of nitrate+Sch promotes full denitrification via the NRFO pathway to produce N_2 while also attenuating N_2O production from incomplete denitrification via the NRAO pathway. These findings indicate that nitrate+Sch combined treatments can be an effective strategy for mitigating N_2O emission through successive Feammox-NRFO cycles.

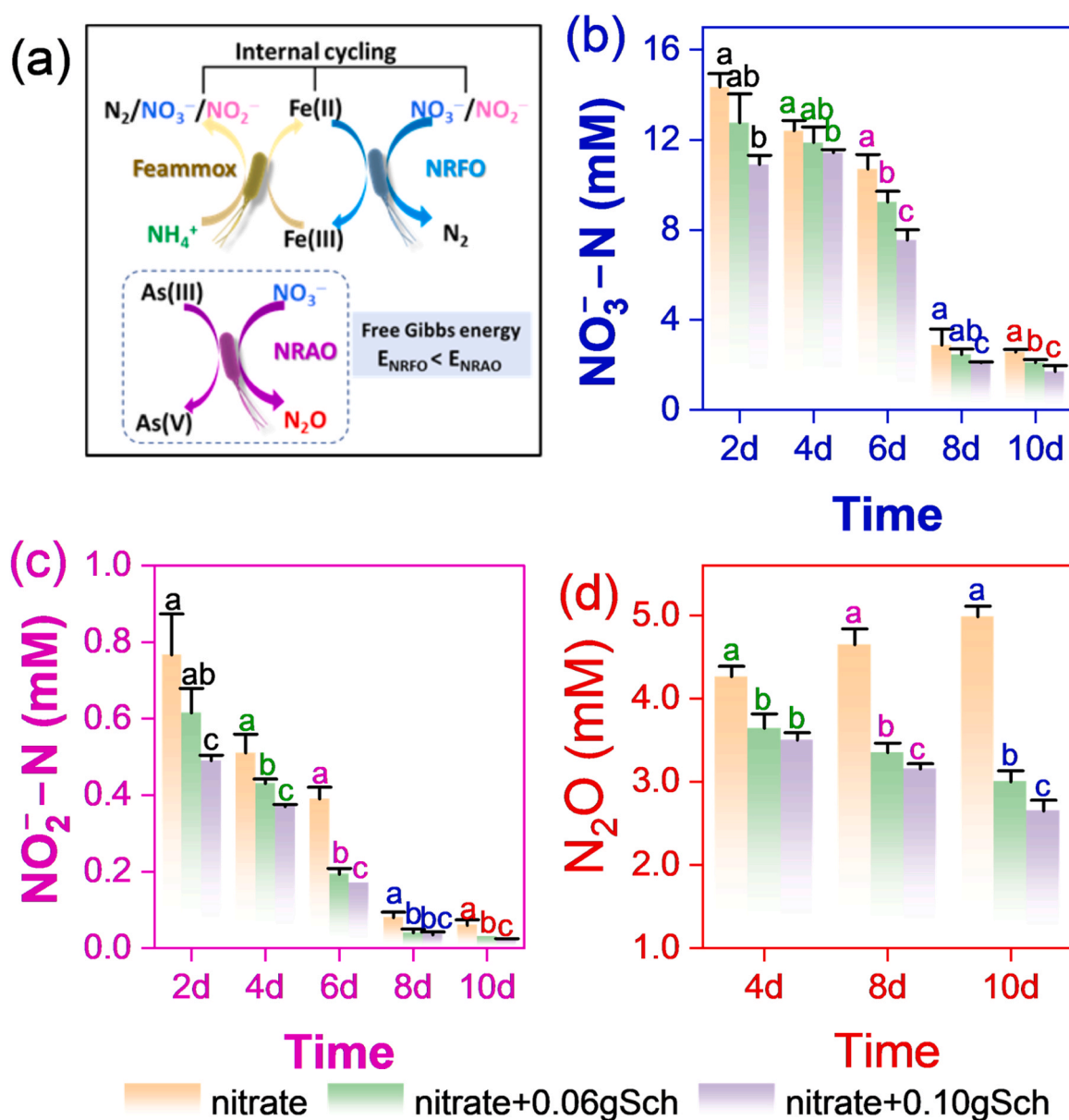


Fig. 2. Conceptual diagram (a) illustrating the N biotransformations involved in the processes of Feammox, NRFO and NRAO in flooded paddy soils ($n = 3$). Associated N concentrations (mean $\pm$ SD) for NO_3^- -N (b) and NO_2^- -N (c) in the flooded layer and emitted N_2O concentrations (d) in the headspace of soil microcosms. Different lower-case letters indicate treatments are significantly different at $P < 0.05$. The error bars represent SD of three replicates.

3.3. Biotransformation of organic C linked to the production of CH_4 and CO_2

In nitrate-supplemented assays, TOC loss escalated with increasing Sch dosage (Fig. 3a), suggesting that Sch promotes either biodegradation of labile organic carbon [31] or complexation of labile DOM with the mineral phase (Sch) [32]. The loss of CH_4 resulting from AOM comprises the pathways of SAOM, DAOM, AOM-FeR and AOM-AsR. Because AOMs interacting with nitrate/nitrite are thermodynamically favored among the various AOM pathways (Table S1), the supplementation of nitrate improved the AOM performance for attenuating CH_4 emission. After 10 days of incubation, production of CH_4 (250.9 and 225.9 mM) from nitrate+Sch (0.06 and 0.10 g) treatments declined by 19–26 % versus the nitrate-alone treatment (Fig. 3b). Notably, the concentrations of nitrate and nitrite among all nitrate-supplemented assays declined to low levels (2.09–2.69 mM and 0.08–0.35 mM; Fig. 2b-c) after incubating for 8 days, subsequently ensuring inefficient DAOM. However, enhanced AOM occurred in nitrate+Sch treatments

(0.06 and 0.10 g) on the 10th day, as less CH_4 was detected (251 and 226 mM; Fig. 3b). This improved AOM performance might result from Sch-associated AOM synergistically enhancing the AOM process under nitrate-depletion conditions. For example, as Sch contains electron acceptors (Fe(III) and SO_4^{2-}) available to particulate in AOM-FeR and SAOM, the co-existence of Sch and nitrate boosts AOM reactions through promoting AOM-FeR and/or SAOM under nitrate-depleted conditions. This inference was supported by the mineralogical characterization and occurrence of S^{2-} in Sch-amended assays (discussed in Section 3.4).

CO_2 is primarily produced from the decomposition of labile DOM by DMRB and in AOM by methane-oxidizing bacteria or ANME [33]. The nitrate+Sch treatments suppressed CO_2 emission with greater suppression at higher Sch dosages (Fig. 3c). Compared to the nitrate-alone treatment on the 10th day, the CO_2 emission from the nitrate+Sch treatments declined by 6.6–7.4 %. Under flooded-anoxic conditions, the decomposition of labile DOM and AOM primarily contributes to CO_2 production [34]. Theoretically, the stimulated TOC consumption and AOM performance resulting from nitrate+Sch treatments (Fig. 3a-b)

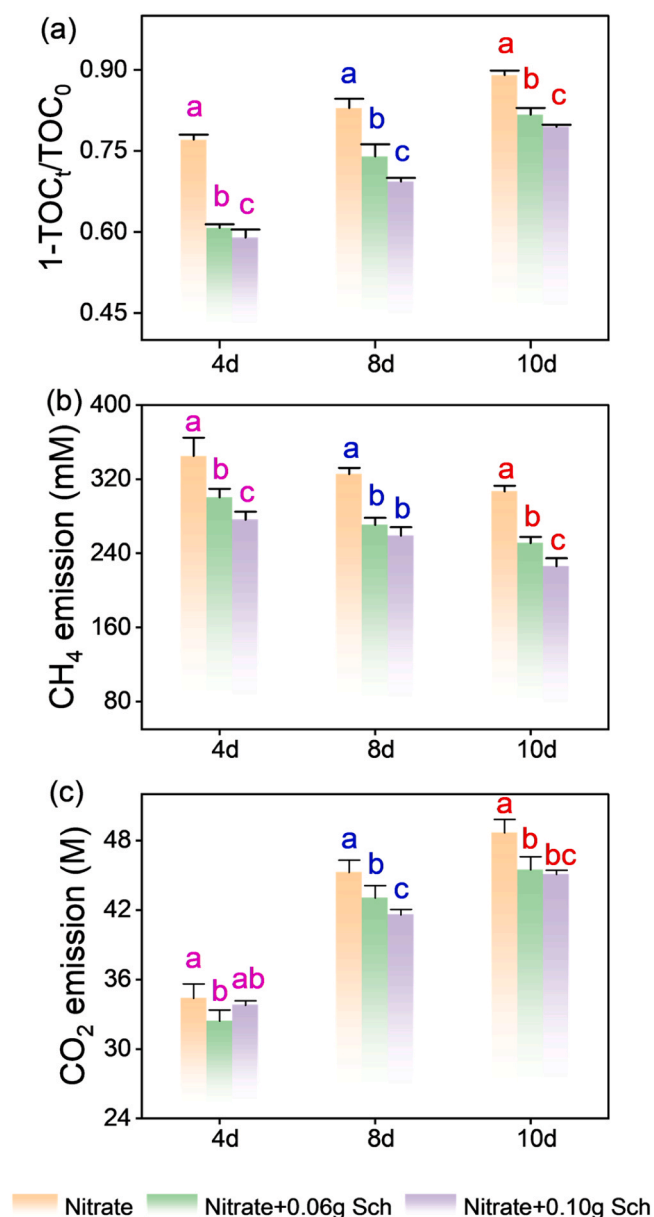


Fig. 3. Removal of TOC in flooded layer (a), and emissions of CH₄ (b) and CO₂ (c) in headspace of soil microcosms ($n = 3$; mean $\pm$ SD). TOC₀ and TOC_t are the DOC concentrations at time 0 and t. Different lower-case letters indicate treatments are significantly different at $P < 0.05$. The error bars represent SD of three replicates.

suggest elevated CO₂ emission. However, the suppression of CO₂ emission from nitrate+Sch treatments are contrary to this premise. Since XPS analysis revealed C 1s signals consistent with carbonate species on the surface, it is plausible that the surplus CO₂ was sequestered in the form of insoluble metal carbonates [35]. This finding also raises further questions regarding how the combined nitrate and Sch treatment influences the microbial community structure, functional gene expression, and key enzyme activities related to carbon and nitrogen cycling [36].

3.4. Characterizations of Sch after incubation

To examine As(III) immobilization and mineralogical alterations of Sch, TEM and XPS were employed to analyze the species of As, Fe, C, O and S after incubation reactions (Fig. 4). TEM-mapping confirmed that As(III) was adsorbed to the surface of Sch particles (Fig. 4a). Specifically, the larger integral area for the fitted peak of As(III) (44.6 eV) versus As

(V) (45.4 eV) [37] appeared in the As 3d spectra (56 % vs. 44 %; Fig. 4b and Table S6). This indicates that Sch-induced As(III) adsorption outperformed the NRAO reaction for immobilization of dissolved As(III) in the flooded layer. By contrast, there was little change in the distribution of Fe(II) and Fe(III) (peaks located at 714.9 and 711.1 eV) [38] within Sch before and after reaction (Fig. 4c). This indicates that coexistence of nitrate was crucial for sustaining the composition of Fe in Sch through replenishment/reoxidation of Fe(II), thereby limiting microbial Fe(III) reduction. After reaction, the signals occurring at 284.8, 286.6, 288.3 and 289.1 eV belong to the binding energies of C-H, C-O, C=O and CO₃²⁻ groups [39] in the C1s spectra (Fig. 4d). These peaks reveal the generation of organic C derived compounds and carbonate deposited on the surface of Sch particles. These new compounds might be derived from microbially synthesized organic C (MOC) compounds and metal carbonates linked to CO₂ produced by AOM [40].

The occurrence of H₂O, -OH and O²⁻ groups at 531.9, 531.2 and 530.0 eV [12] in O1s spectra (Fig. 4e) indicates shifts in the oxygen-bearing groups resulting from incubation reactions. The content of adsorbed water (H₂O) varied from 0 % to 29.4 % (Table S6) after reaction providing direct evidence for bulk complexation of Fe(II)/Fe(III)-(hydr)oxides. Moreover, the generation of sulfur-bearing groups such as SO₃²⁻, S₂²⁻ and S²⁻ (associated with the binding energy of 165.7, 162.4 and 161.3 eV) in S2p spectra (Fig. 4f) implies reduction of SO₄²⁻ (168.6 eV) in the Sch, which likely results from the SAOM reaction producing S²⁻. The production of S₂²⁻ and S²⁻ associated with the SAOM reaction might result in formation of sulfide precipitates. An increase in S²⁻ concentrations occurred in the Sch treatments, whereas no S²⁻ was detected in nitrate+Sch treatments (Fig. S6).

3.5. Activities of key enzymes involved in denitrification, methanogenesis and AOM

Denitrification processes (autotrophic/heterotrophic) consume electrons by a series of denitrifying reductases (NAR, NIR, NOR and NOS, shown in Fig. 5a) involved with the different steps of nitrogen reduction [19]. For DAOM-associated denitrification, the activity of Mcr largely influences the oxidation of CH₄ in a reverse methanogenesis pathway (Fig. 5b) [41]. Over the course of the 10d incubation, a notable decline in the residual nitrate concentration was observed on the 8d, implying that the activities of denitrifying enzymes reached their maximum levels. In this study, activities of the four denitrifying enzymes increased as the dosage of Sch increased in the nitrate+Sch assays (Fig. 5c). The activity of the NOS enzyme responsible for the reduction of N₂O to N₂ increased by 20.7 % in the nitrate+Sch (0.10 g) assay compared to the nitrate-alone treatment. The increased activity of NOS resulted in lower production of N₂O from the nitrate+Sch assays (Fig. 2d). The improved denitrification performance might be attributed to a higher level of extracellular Sch reduction-derived Fe(II). This may have a stimulating effect on the activities of denitrifying enzymes due to competitive uptake of the available electron donor, Fe(II), that is redox reactant in autotrophic denitrification reactions [42]. In flooded paddy soils, autotrophic/heterotrophic denitrification pathways are initiated by denitrifying enzymes utilizing various electron donors (e.g., As(III)/Fe(II) and labile DOC). In the absence of sufficient labile DOC, heterotrophic denitrification (estimated from DOC consumption; Fig. 3a) is hindered and NRFO has a higher thermodynamic feasibility than NRAO (Eq. 9 vs Eq. 10 in Table S1). Hence, microorganisms could preferentially utilize Fe(II) as an electron donor for full denitrification to completely reduce nitrate to N₂. This full denitrification process requires an energy investment that preferentially utilizes Fe(II) to meet its metabolism requirements.

Methane dynamics in flooded paddy soils are modulated by methanogens and methanotrophic microorganisms wherein the Mcr-mediated enzyme catalytic process serves as the key intermediary balancing CH₄ production versus oxidation [26,43]. In the presence of nitrate, Mcr-mediated CH₄ oxidation during the initial step of AOM by ANME

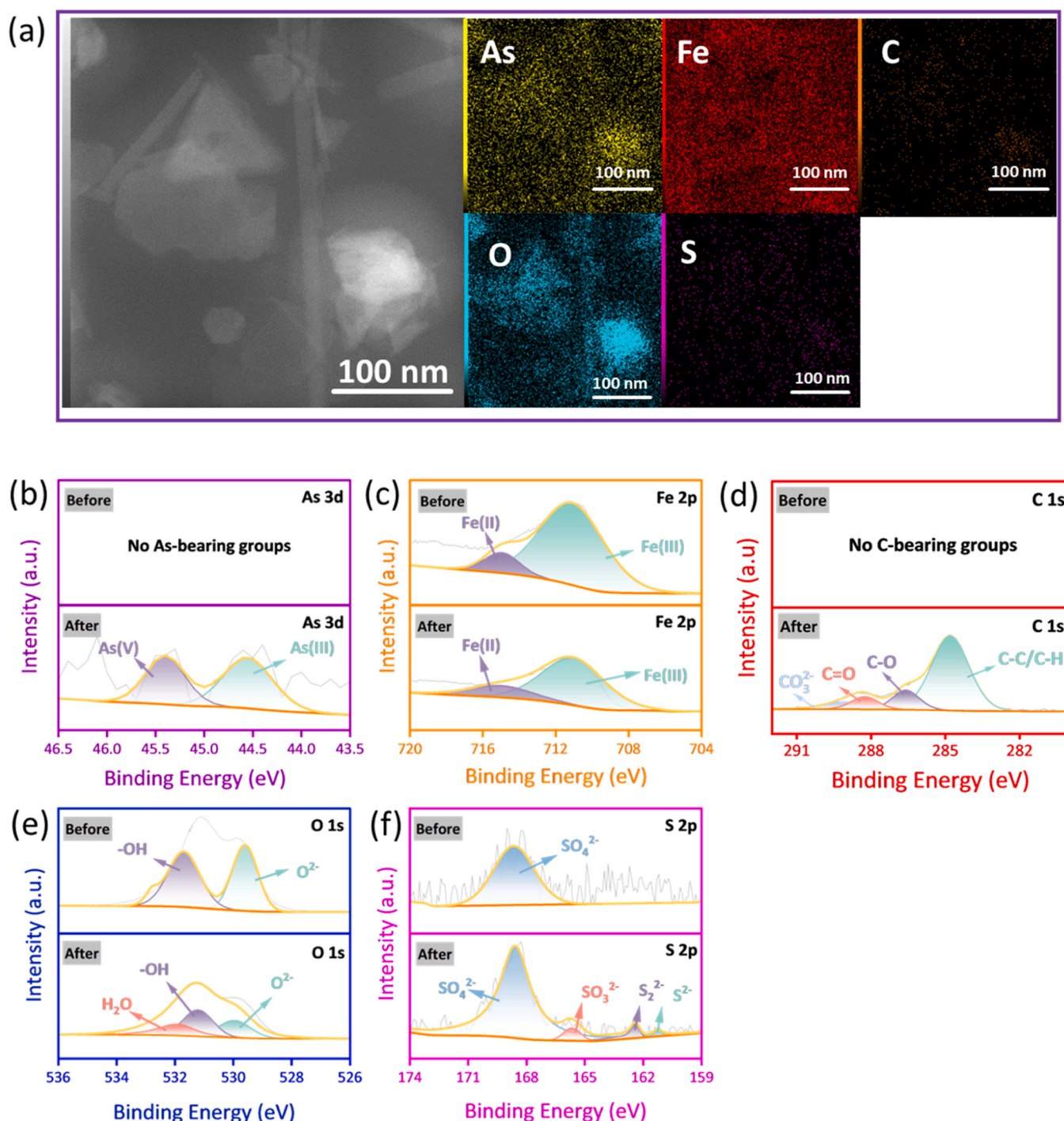


Fig. 4. TEM morphology (a) of Sch particles and corresponding EDS maps of As, Fe, C, O, and S distribution. XPS spectra showing speciation of adsorbed As (b), Fe (c), C (d), O (e), and S (f) on the Sch particles separated from dialysis bag on the 10th day-incubation.

commonly outperforms CH₄ production by methanogens [44]. ANME initiated by Mcr also exhibits a high affinity for various electron acceptors (e.g., Fe(III) and SO₄²⁻) within AOM [44]. This implies that AOM performance will be amplified in the presence of nitrate and Sch due to co-occurrence of AOM-FeR and SAOM. This inference is supported by the activities of Mcr that were up-regulated by 13.4 % and 23.3 % in the nitrate+Sch (0.06 and 0.10 g) treatments versus nitrate-alone (Fig. 5c). Further support is provided by the increased S²⁻ production in the Sch-alone treatments (Fig. 4f and Fig. S6) implied sulfate in Sch particles contribute to Mcr-mediated SAOM while the suppressed S²⁻ production in the Sch+nitrate treatments might due to the released S²⁻

coprecipitate with mobile As(III) form poorly soluble As₂S₃ precipitates.

3.6. Changes in composition and functional genes of microbial communities

3.6.1. Characteristics of the microbial community

Metagenomic sequencing revealed that *Pseudomonadota* (20–77 %), *Myxococcota* (7–53 %), *Thermodesulfobacteriota* (1–13 %), and *Actinomycetota* (1–7 %) were the dominant phyla among bacteria, whereas *Euryarchaeota* (2–5 %) was the dominant phylum for archaea across all treatments (Fig. 6a). Specifically, nitrate input prominently enriched the

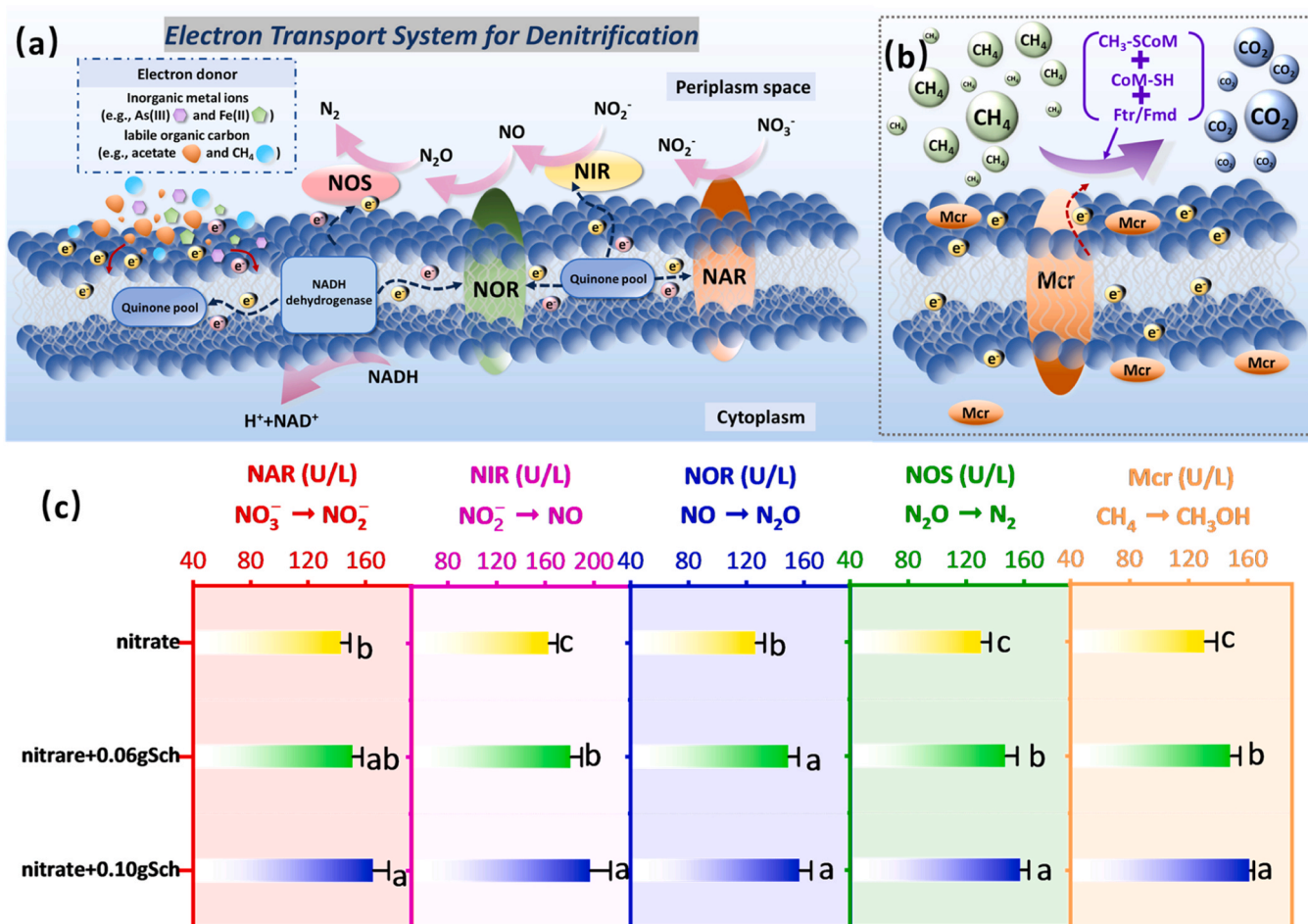


Fig. 5. Schematic diagrams of electron transport as modulated by denitrifying enzymes (a) and the nitrate-dependent AOM process mediated Mcr enzyme (b). Activities of denitrifying enzymes and CH_4 oxidation enzyme (Mcr) determined on 8th day incubation samples (c) ($n = 3$; mean $\pm$ SD). Different lower-case letters indicate treatments are significantly different at $P < 0.05$. The error bars represent SD of three replicates.

abundance of *Pseudomonadota* and decreased the abundance of *Myxococcota*. Meanwhile, *Euryarchaeota* incorporating the majority of methanogens distinctly decreased with the addition of foreign electron acceptors (nitrate and/or Sch). This provides a mechanist explanation for the lower CH_4 production in the nitrate and/or Sch treatments (Fig. 3b).

Key genera in the archaea and bacteria domains were categorized based on their functions in regulating processes associated with C/N/S and Fe/As cycles (Fig. 6b). The bubble plot of RPKM abundances for genera indicates that the distribution of metal-reducing bacteria responsible for As(V)/Fe(III) reduction was dominant in CK and Sch treatments and their abundances were enriched upon addition of Sch. Owing to the improved bioavailability of Fe(III) and SO_4^{2-} contributed by Sch-supplemented assays, the addition of Sch fostered enrichment of FRB (e.g., *Geobacter*, *Sphingomonas*, and *Anaeromyxobacter*) and SRB (*Desulfuromonas*, *Desulfosarcina*, and *Desulfovibrio*). Correspondingly, this increased the extent of dissimilatory Fe(III) and SO_4^{2-} reduction that might indirectly facilitate the mobilization of As in flooded paddy soils (Fig. 6b) [32]. Conversely, the input of nitrate (with/without Sch) induced an opposing impact on the enrichment of metal-reducing bacteria and SRB while promoting the enrichment of autotrophic NRFO and NRAO bacteria (e.g., *Streptomyces*, *Pseudomonas*, *Stenotrophomonas*, and *Azoarcus*). Since nitrate sustains the metabolism of NRFO and NRAO bacteria, the addition of nitrate accelerated the generation of Fe(III)/As(V) in nitrate+Sch assays, which in turn favored As(III) immobilization through adsorption, complexation, and coprecipitation [45,46].

Emissions of N_2O and CH_4 strongly depend on the bioavailability of

nitrate and/or Sch to different microorganisms (Fig. 6b). The increased abundance of specific anammox bacteria (e.g., *Candidatus Methylophilus* and *Candidatus Brocadia*) with the input of nitrate and Sch alone than CK group in our study infers the occurrence of anammox, which attenuates N_2O production. Nevertheless, we posit that full denitrification linked to Fe(II) oxidation might be the dominant process responsible for the decline of N_2O emission from the nitrate+Sch treatments. As NRFO bacteria had a higher abundance than anammox bacteria (Fig. 6) and nitrite was nearly depleted after 4 days (Fig. 2c), the conditions for anammox became less favorable as the incubation time proceeded. Since the nitrate+Sch treatment supplied the essential substrates to support the enrichment of NRFO bacteria (e.g., *Streptomyces*, Fig. S7) and activation of enzyme activities (Fig. 5c), full denitrification performance linked to Fe(II) oxidation is thermodynamically feasible to favor the ultimate production of N_2 versus N_2O .

We found that the emission of CH_4 was influenced by both methanogenesis and AOM. Based on the overall abundance of archaeal genera, the addition of nitrate led to a decreased abundance of methanogens whereas it increased the abundance of ANME (Fig. 6b). AOM is carried out by ANME and bacteria or specific ANME-bacteria consortia that utilize various electron acceptors [44]. Our study confirmed that the dominant archaeon, *Candidatus Methanoperedens*, was the most abundant ANME genus in the nitrate+Sch treatment. *Candidatus Methanoperedens* is capable of catalyzing nitrate/Fe(III)-dependent AOM via "reverse methanogenesis" and putative extracellular electron transfer pathways [47]. Hence, the enrichment of *Candidatus Methanoperedens* provides a metabolic pathway for effectively promoting AOM, thereby

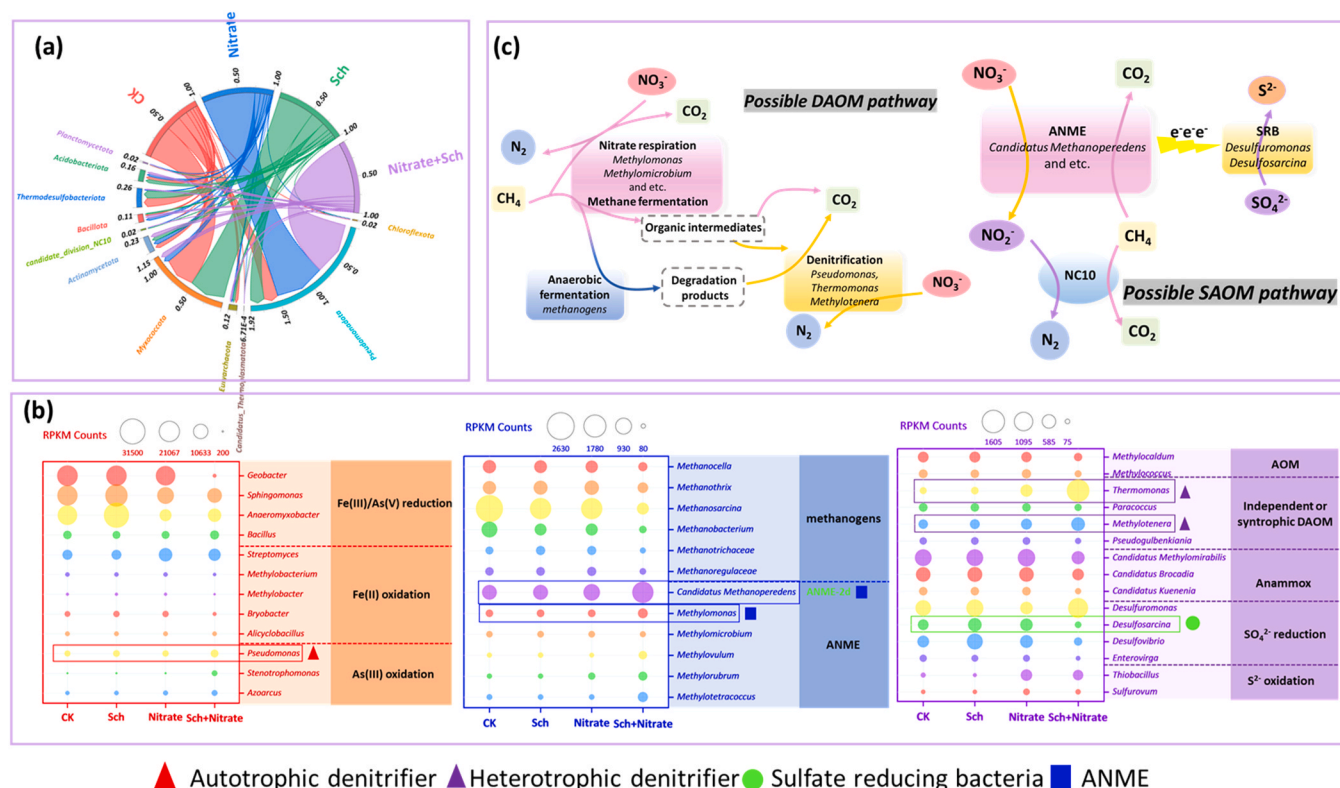


Fig. 6. Distribution of microbial community at phylum level (a), the RPKM (reads per kilobase million) abundances of key genera responsible for As/Fe/C/N/S biotransformations under different amendments (b), and possible AOM pathway regulated by key microorganisms (c). Dosages in Sch-alone and Sch-nitrate treatments are both 0.10 g.

attenuating CH_4 emission from nitrate+Sch treatments.

Autotrophic *Pseudomonas*, as well as heterotrophic *Thermomonas* and *Methylothermobacter* (Fig. 6b), were the major denitrifiers involved in the syntrophic DAOM process and they displayed greater abundances in nitrate+Sch treatments than with other amendments. Notably, *Pseudomonas*, *Thermomonas* and *Methylothermobacter* are reported as important methylothermotrophic denitrifiers that collaborate with *Methylothermobacter* (ANME-2d) in syntrophic DAOM (Fig. 6c). In this process, *Methylothermobacter* oxidizes CH_4 to methylic organics that are subsequently utilized by *Pseudomonas*, *Thermomonas* and *Methylothermobacter* to convert nitrate/nitrite to N_2 [48,49]. We also found that *Methylothermobacter* was consistently more abundant in nitrate+Sch treatments than with other amendments (Fig. S7e). Moreover, *Desulfuromonas*, a typical SRB cooperating with ANME-2 archaea that are reported to participate with AOM-FeR [50], was particularly enriched in the nitrate+Sch treatments (Fig. S7g). Hence, the enrichment of key microorganisms resulting from nitrate+Sch treatment fueled syntrophy in specific ANME-bacteria consortia to support multiple AOM pathways. Together, the enrichment of these key microorganisms in specific ANME-bacteria consortia of nitrate+Sch treatments was essential for sustaining highly efficient methane fermentation pathways that effectively fuel the consumptions of CH_4 and N_2O in anoxic-flooded paddy soils.

3.6.2. Genomic-level links to integrated As/Fe/N/C/S cycles

To further clarify the integrated mechanisms of microorganism-mediated As/Fe/N/C/S cycling, the relative abundances of key genes involved in As/Fe/N/C/S metabolic processes (Tables S7-S8) as annotated by the KEGG Orthologs database were collectively analyzed (Fig. 7a). Reductive dissolution of Fe(III) and As(V) is commonly considered active under most flooded anoxic conditions [51], whereas microbial oxidation of Fe(II) and As(III) is only considered active under selective anoxic conditions [17]. Accordingly, we found that the abundance of genes involved in Fe(III)/As(V) reduction was higher than

those of Fe(II)/As(III) oxidation. The addition of nitrate (with/without Sch) markedly increased the abundance of As(III)-oxidizing *aoxAB*, Fe(II)-oxidizing *cox* genes (*coxABC*) and *cta* genes (*ctaCDE*), as well as denitrifying genes, highlighting that nitrate was an important factor driving the transcription of key functional genes involved in NRFO and NRAO. Conversely, the abundance of genes encoding anammox (*hns*) and sulfate reduction (*apr*, *cys*, and *phs*) were particularly up-regulated in Sch-alone treatment. Compared with the CK group, the abundance of key genes involved in regulating AMO (*pmoA*) and methanogenesis (*mcr*) pathways was increased and decreased with the additions of nitrate and Sch, respectively. Variations in the abundance of As/Fe/C/N/S cycling genes revealed the differential microbial processes influenced by nitrate and Sch.

To enhance comparisons, the proportions of defined metabolism pathways within the total transcription of active As/Fe/N/C/S biogeochemical processes were analyzed by integrating total abundances of related genes encoding for the same metabolism pathway (Fig. 7b). The high proportion (49–87 %) of denitrifying genes associated with N cycling processes infers that denitrification was a major microbially-mediated N cycling process. The highest proportion (87 %) of N-related genes occurred in the nitrate+Sch treatment that promoted the strongest denitrification performance resulting in complete denitrification to N_2 . Since Sch supplies abundant sulfate for microbial reduction, the genes encoding for sulfate reduction accounted for a higher proportion of S redox related processes in Sch assays (91–92 %) compared to non-Sch assays (72–79 %).

Carbon biotransformation processes comprising methanogenesis, AMO, C fixation and C degradation are considered active in flooded paddy soils [52–54]. The relative abundance of genes involved in C fixation was highest (76–81 %), whereas the abundance of genes linked to methanogenesis (1–6 %), AMO (2–11 %) and C degradation (11–15 %) were considerably lower. The high abundance of C fixation genes signifies a large C-sequestration potential associated with

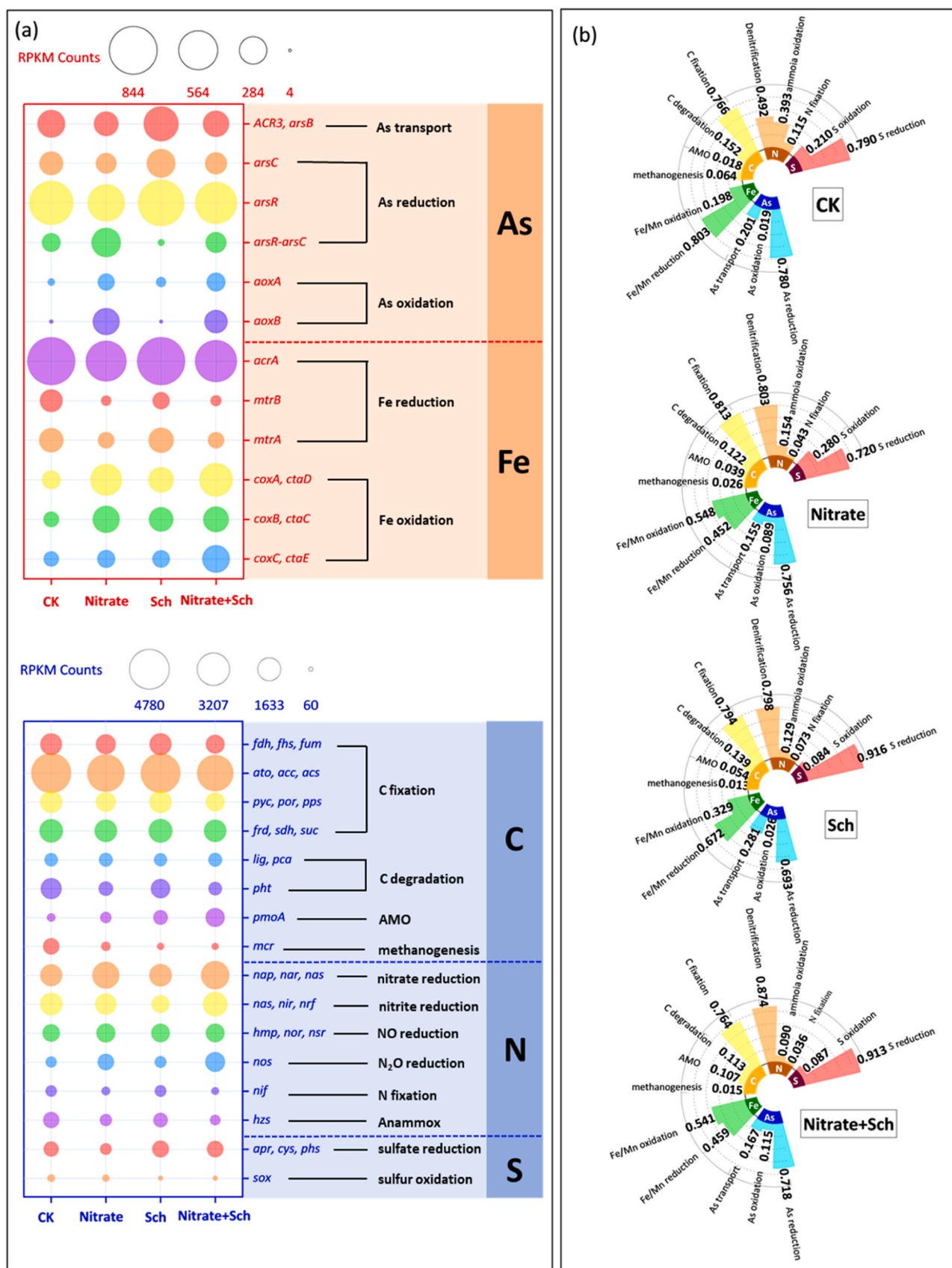


Fig. 7. RPKM abundances of genes associated with As-Fe-C-N-S cycling (a) and proportions of functional genes identified for a defined elemental biotransformation pathway (b). Dosages in Sch-alone and Sch-nitrate treatments are both 0.10 g.

autotrophic microorganisms. The nitrate+Sch treatments achieved the highest abundance of AMO genes (11 %) among all treatments (2–11 %), consistent with the observed AMO performance. Moreover, the up-regulated transcription and high relative abundance of genes involved in denitrification and sulfate reduction were consistent with that of the AMO gene in nitrate+Sch treatments, further highlighting that AOM was enhanced by integration with denitrification and sulfate reduction.

3.7. Mechanisms for simultaneous suppression of As(III) mobilization and GHG emissions

Mantel tests incorporated the contents of As and Fe, GHG emissions (CO_2 , CH_4 , N_2O), NO_3^- , NH_4^+ , and TOC as the environmental variables. The analyses were conducted using the mantel function in the vegan R package, with statistical significance assessed based on 999 permutations. Mantel analysis confirmed that residual As(T) exhibited a strong correlation with Fe(T) mobilization ($R=-0.67$, $P<0.05$) and residual nitrate concentration ($R=0.96$, $P<0.01$) in nitrate+Sch treatments (Fig. 8). Similarly, Mantel analysis revealed that N_2O emission was closely linked with nitrate, ammonia and TOC concentrations, suggesting that processes related to these constituents contributed to N_2O emission. In particular, the positive correlation between N_2O production and the concentrations of nitrate loss and Fe(II) generation provides direct evidence for NRFO-derived full denitrification in nitrate+Sch treatments fostering the removal of N_2O . Moreover, CO_2 production exhibited a negative correlation with TOC ($R=-0.97$, $P<0.01$) and CH_4

($R=-0.93$, $P<0.01$) indicating suppression of fermentation-dominated methanogenesis and active AOM production of CO_2 .

The nitrate+Sch treatment achieved the desirable objectives of As(III) immobilization and GHG emission reductions in flooded paddy soils. Based on a synthesis of our results, the main mechanisms are profiled in Fig. 9. The interactions between microbes, nitrate and Sch in flooded paddy soils induced integrated responses leading to changes in selective allocation patterns and utilization efficiencies of various substrates. The mechanisms for attenuation of As(III) pollution comprised enhanced immobilization of As(III) in the flooded layer and suppressed reductive dissolution of As(V) in sediments. The coexistence of nitrate and Sch favored the integrated redox cycles involving Sch-derived Fe through successive Feammox and NRFO. This leads to regeneration of Fe(III)-oxides/oxyhydroxides that fosters immobilization of dissolved As(III) through adsorption, complexation, and coprecipitation [10]. Owing to NRFO having a higher thermodynamic favorability than NRAO, full denitrification by NRFO displaced NRAO, thereby attenuating N_2O emission. Likewise, AOM-FeR competitively outperformed AOM-AsR, effectively blocking the reductive dissolution of As(V) in sediments. Moreover, the S^{2-} generated from SAOM is available to coprecipitate with dissolved Fe(II) and As(III) to form FeS and As_2S_3 precipitates [55], providing another pathway for As(III) immobilization.

To mitigate GHG emissions, the incorporation of nitrate and Sch into flooded paddy fields provides essential substrates to promote the simultaneous occurrence of AOM, denitrification and anammox owing to co-utilization of multi-electron acceptors/donors. The surplus nitrate not only promoted reliable As(III) immobilization, but also fostered full

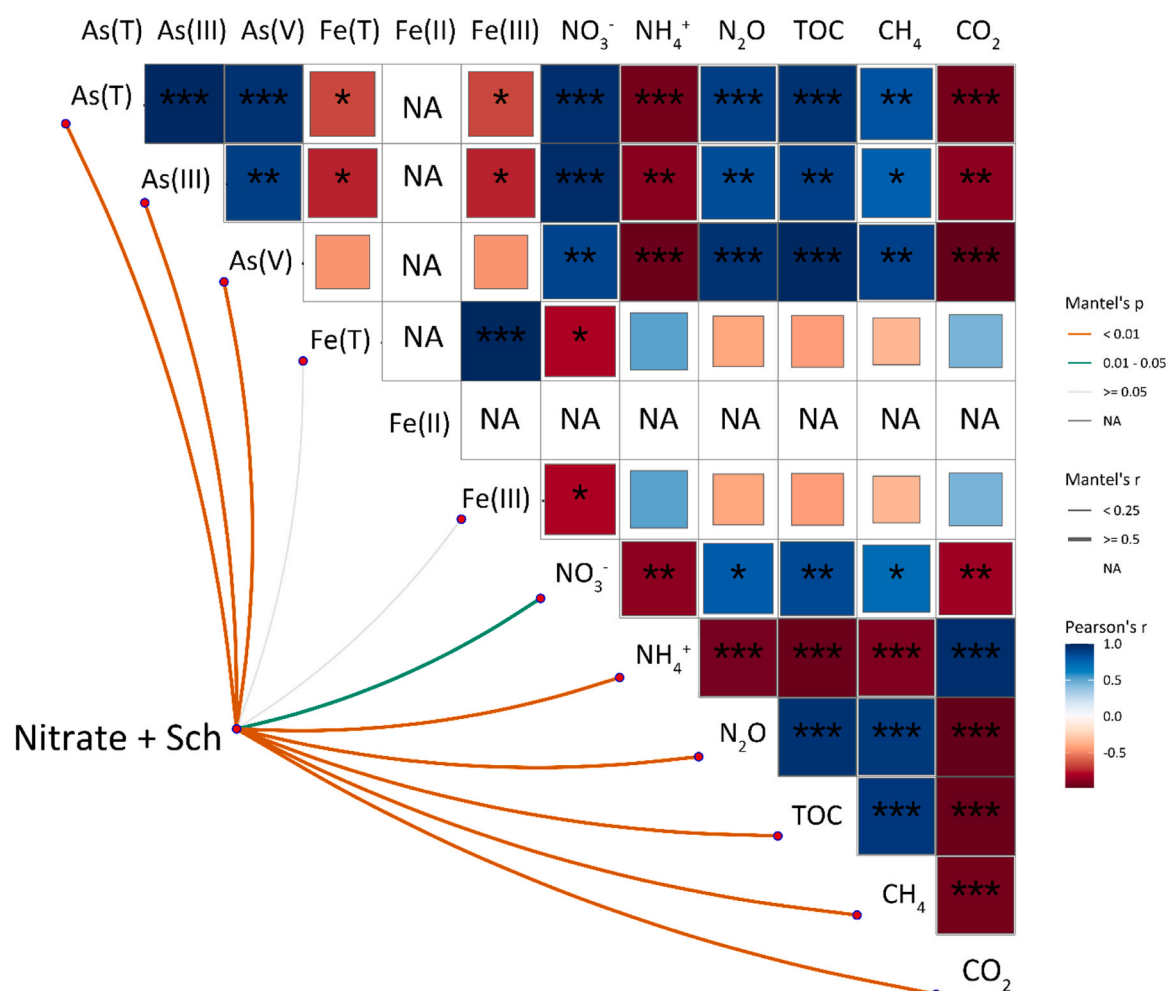


Fig. 8. Mantel-test correlations to identify relationships of among major redox parameters in the Sch+nitrate combined amendment.

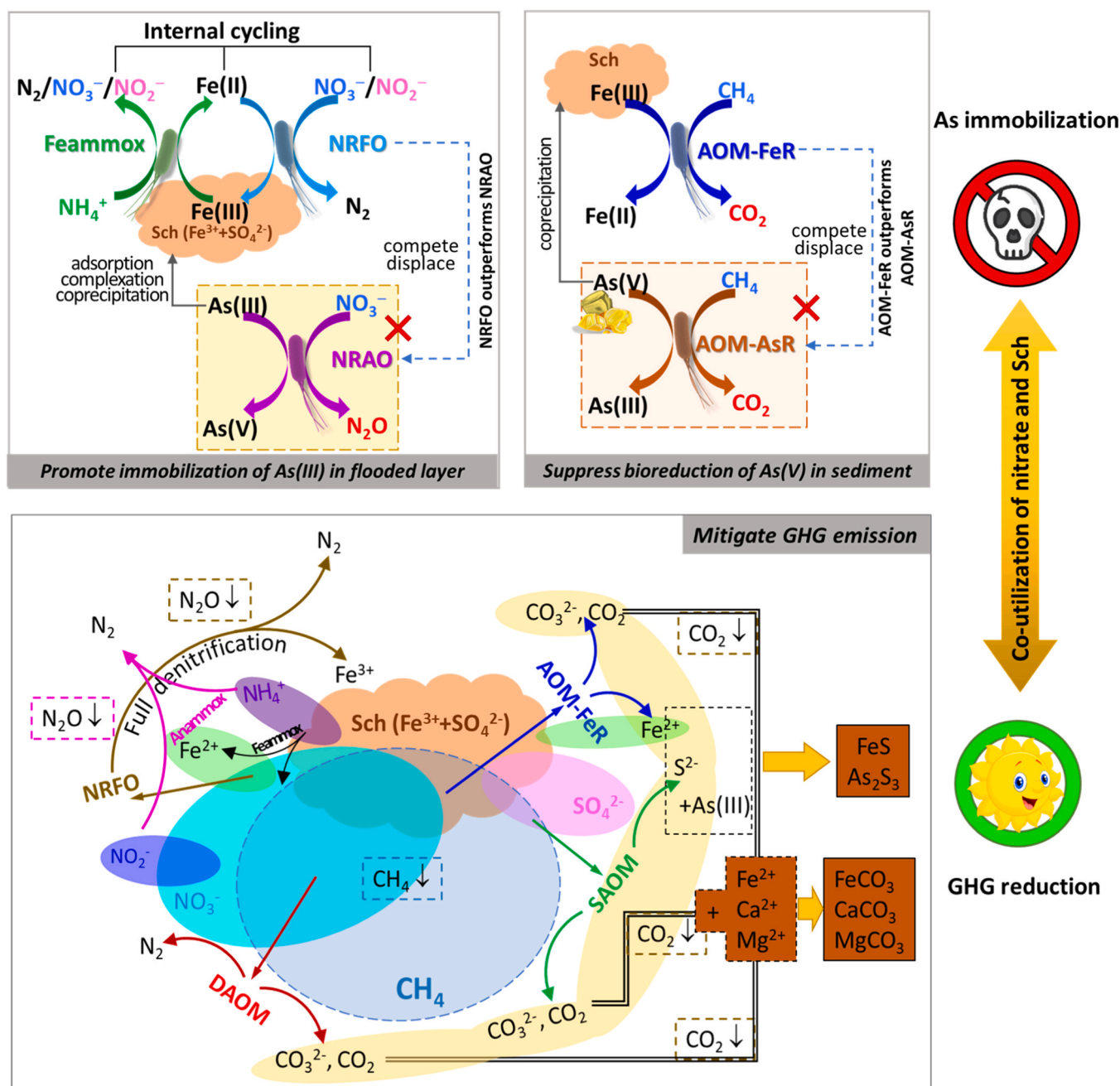


Fig. 9. Schematic illustration of the integrated mechanisms contributing to the simultaneous immobilization of As(III) and mitigation of GHG emissions in flooded paddy soils by the Sch+nitrate amendment.

NRFO-derived denitrification and/or anammox to attenuate N_2O emission. Since nitrate is a thermodynamically favorable electron acceptor for AOM among the various electron acceptors in anoxic soils, the presence of nitrate effectively decoupled the AOM-AsR pathway, thereby eliminating the negative feedback of As(III) mobilization and reinforcing AOM. These exploitative strategies for competing substrate resources highlight the importance of nitrate amendment for attenuating As(III) mobilization and GHG emissions.

Integration of the N and S cycles with Fe(III) reduction affected AOM in the nitrate+Sch treatments by providing multiple-linkages within the AOM network that intensified the consumption of CH_4 . Some of the CO_2 resulting from AOM was dissolved as carbonate, which can then coprecipitate with dissolved Fe(II), Ca(II) and Mg(II) to form poorly soluble carbonate precipitates. This provides an active pathway for CO_2 sequestration in the form of carbonate minerals, which could effectively

mitigate the emission of atmospheric CO_2 . Overall, the surplus Sch and nitrate resulted in a complex interplay between diverse microbial interactions responsible for regulating the integrated As/Fe/C/N/S cycles through resource interference, competition, and cooperation mechanisms. Understanding these mechanistic pathways provides the fundamental information necessary to design practical strategies for simultaneously attenuating As(III) mobilization and GHG emissions.

4. Conclusions

This study demonstrates the efficacy of nitrate+Sch amendments for suppressing As(III) mobilization and GHG emissions from flooded paddy fields versus the shortcomings of treatment with nitrate or Sch alone. The combined treatment allows the regeneration of Fe(III)-oxides/oxyhydroxides via coupling with Feammox and Fe(II)-derived

denitrification under NH_4^+ -enriched conditions. These mechanisms favored the adsorption, complexation, and coprecipitation of dissolved As(III) and attenuated the bioreduction of Fe(III)/As(V) in sediments. The combined treatment activated the microbial transformations involved in full denitrification and AOM, wherein N_2O was completely reduced to N_2 while CH_4 was oxidized to CO_2 . Moreover, XPS analysis suggested the formation of carbonate surface species, while bulk mineral precipitation requires further verification. Overall, our findings demonstrate the efficacy of nitrate+Sch amendments as a remediation strategy to address As pollution and GHG emissions in flooded paddy fields.

CRediT authorship contribution statement

Ruotong Mao: Software. **Shuyun Zhang:** Formal analysis. **Zilu Ye:** Resources. **Wenhui Ma:** Resources. **Zheng Chen:** Writing – review & editing, Supervision. **Siwen Xia:** Writing – review & editing, Supervision. **Yilin Chen:** Writing – original draft, Software. **Randy A. Dahlgren:** Writing – review & editing. **Hui Gao:** Resources, Funding acquisition. **Jing Zhang:** Investigation. **Honghui Wang:** Methodology. **Xiyu Zhao:** Writing – original draft, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jece.2025.119845](https://doi.org/10.1016/j.jece.2025.119845).

Data availability

Data will be made available on request.

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