

# Nitrate alleviate dissimilatory iron reduction and arsenic mobilization by driving microbial community structure change

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## ARTICLE INFO

### Keywords:

Nitrate amendments  
Arsenic  
Paddy soil  
Dissimilatory iron reduction

## ABSTRACT

Anaerobic situation induced by long-term flooding of paddy soils makes dissimilatory iron (Fe) reduction and Arsenic (As) reduction in pore water. In our research, different dosage of nitrate (1 and 20 mmol NO<sub>3</sub><sup>-</sup> kg<sup>-1</sup> soil, LN and HN, respectively) were used as amendments for As immobilization. The effect on soil properties such as pH, Eh, sulfate, and nitrate; the effect on As(III) and dissimilatory iron reduction and the effect on microbial community structure were investigated. Our results showed that With the addition of nitrate, the reduction of Eh was accelerated. Especially for HN, the Eh decreased from 139 to 40 mV in 30 days, is higher than Eh in original soil (CK). The dissimilatory iron reduction was significantly depressed by the addition of nitrate, Fe<sup>2+</sup> in nitrate amendments are much less than control, especially for HN, Fe<sup>2+</sup> concentration was 62.58% lower than control. While Final As(III) concentrations were 284.67, 223.87, and 190.70mg Kg<sup>-1</sup> for CK, LN, and HN treatments, respectively. In both LN and HN, the concentration of NO<sub>3</sub><sup>-</sup> faded with incubation time, which means that NO<sub>3</sub><sup>-</sup> could act as an electron acceptor instead of Fe<sup>3+</sup> and As(V). Moreover, nitrate has selectivity for microbes, while the abundance of *Clostridia* and *Geobacteraceae*, which play a major role in the reduction of dissimilatory iron, is strongly inhibited, thereby inhibiting the process of dissimilatory iron reduction. Our results showed that by promotion of decreasing Eh, inhibition dissimilatory iron reduction, and Arsenic speciation transformation, nitrate could act as an effective amendment for As immobilization in paddy soils.

## 1. Introduction

According to the Report on the National Soil Contamination Survey of China, 2.7% of soils were contaminated with arsenic (As) [1]. Continuously submerged flooding soils strongly influenced the bioavailability of As, resulting in As bioavailability in flooded paddy soils is much higher than aerobic soil [2–4]. In particular, paddy soils in southern China has been suffering more serious As contamination with total soil As ranging from 69 to 28,522 mg kg<sup>-1</sup> [5], resulting in elevated levels of As in rice grain that may pose a significant risk to public health for populations consuming rice as the staple food [6–8]. It is critically important to keep the food security of rice, especially for South and Southeast Asia such as China, of which rice is the main food resource [9].

Microbes played a key role in the driven bioavailability of arsenic in paddy soils. In general, As is mobilized mainly as arsenite (As(III)) as a

result of reductive dissolution of iron (oxyhydr)oxides [10–12]. Of which Fe reducing bacteria(FRB) dissolved Fe (hydro)oxides by using Fe (III) as electron acceptor [13], release arsenic from minerals. Furthermore, low Eh induced by flooding influence arsenic be As(III) instead of As(V) in porewater [14], While microbes including *Halomonas* strain ANAO-440, *Polaromonas* sp. GM1 and *Alkalilimnicola ehrlichii* strain MLHE-1 is proved to participate in speciation transformation of arsenic for detoxification or dissimilatory pathway [15,16]. Thus, the strategies for inhibition of reductive dissolution of iron (oxyhydr)oxides and/or oxidize As(III) to As(V) should be investigated.

There are two main mechanisms to decrease As bioavailability in paddy soils: a. Addition of amendments for sorption mobilized As(III). For example, Fe amendments (such as zero-valent iron (ZVI), ferrous ion, and Fe (hydr)oxides) to afford new sorption site for As(III)/As(V) [17–19]. ZVI-modified biochar will stabilize As by the formation of FeAsOOH complexes [20,21]. b. Alleviate or inhibition of microbial

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<https://doi.org/10.1016/j.surfin.2021.101421>

Received 3 July 2021; Received in revised form 9 August 2021; Accepted 18 August 2021

Available online 26 August 2021

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dissimilatory iron reduction. Sulfate and nitrate were used as electron acceptors instead of Fe(III), thus decrease the release of As in paddy soils [22]. Besides this, nitrate was also found to be an oxidizer for As(III) to As(V) with the mediate of some microbes such as *Acidovorax* strain ST3 [23]. Furthermore, it was reported that there are 200 Kg N ha<sup>-1</sup> (equivalent to 5.5 mmol Kg<sup>-1</sup> soil in the 0-20 cm plow layer, assuming a bulk density of 1.3 g cm<sup>-3</sup>) added to paddy soils in China [23]. Thus, nitrate was an optional amendment that can be used not only as a fertilizer but also as a stabilizer for As contaminated soils.

Despite the influence of nitrate for the As biogeochemical cycle in paddy systems, there are few studies on dynamic microbial community structure change of flooding paddy soils. In the present study, we hypothesized that the addition of nitrate can promote microbes associated with NO<sub>3</sub><sup>-</sup> reduction and As(III) oxidation, and restrain microbes associated with Fe(III) reduction, resulting in inhibition of reductive dissolution of iron (oxyhydr)oxides. Incubation experiments were conducted for 30 d, the dynamic profile of As and Fe speciation, as well as changes in soil As distribution among the labile fractions under flooded conditions with and without nitrate treatment, were investigated. Furthermore, microbial community structure changes during incubation were explored to figure out the influence of nitrate on microbes. The results suggest the possibility of using nitrate to manipulate the biogeochemical cycle of As in paddy soils.

## 2. Methods and materials

### 2.1. Soil sampling and characterization

As contaminated paddy soils are collected from Shan Tou, China in the present study. The soils have been contaminated with As due to nearby mining activities. Soils were air-dried, disaggregated, and passed through a 2 mm sieve before being stored in plastic containers in the dark. Soil properties, including pH, organic matter content, cation exchange capacity (CEC), NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, speciation distribution of Fe (free iron oxide, amorphous iron, and total iron) and As were determined. Soil pH was determined in a soil suspension possessing a soil: 0.01 mol L<sup>-1</sup> CaCl<sub>2</sub> ratio of 1: 2.5 (w/v) with a pH meter. Organic matter content was determined by the ignition method (weight loss at 450 °C) [24]. CEC was measured by the extraction method with ammonium acetate (NH<sub>4</sub>OAc) and potassium chloride (KCl) [25]. NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup> was measured by ion chromatography directly. Fe and As speciation were determined according to methods of previous studies [26].

### 2.2. Incubation experiment

Every 5.0000 g soil was placed in a 100 mL brown serum bottle. 1 and 20 mmol NO<sub>3</sub><sup>-</sup> kg<sup>-1</sup> soil of nitrate (LN and HN, respectively) was added to the bottle. Ultrapure water was added to keep the water: soil mass ratio as 4:1. For comparison, original soil and sterilized soil were submerged by ultrapure water and incubated at the same time (CK and SCK, respectively). Each treatment was put into the anaerobic glove box to circulate nitrogen and vacuum three times to remove the oxygen, sealed and shaken at 220 r/min for 5 min. Incubation in the dark for 1, 5, 10, 15, 20, 25, and 30 d in the anaerobic incubator at 30 °C. 3 serum bottles were prepared, sacrificed sampled and tested at each sampling time. Eh and pH of each sample were tested. Samples were centrifuged, sediments and solution were collected for measurements. NO<sub>3</sub><sup>-</sup>-N and NO<sub>2</sub><sup>-</sup>-N of supernatant were detected by Ion chromatography, while As (III) and As(V) were separated and measured by atomic fluorescence. After air dried, 1 g sediment was saturated in HCl for 24 h at 30 °C, and then centrifuged and measured the Fe<sup>2+</sup>/Fe<sup>3+</sup> concentration in supernatant. All samples have 3 replicates.

### 2.3. Analysis of soil microbial properties

Genomic DNA of soil microbe was extracted from previously

homogenized and thawed materials removed from their mesh bags using the Fast DNA® SPIN Kit for Soil (MP Biomedicals, France) according to the manufacturer's instructions with the following modifications. Total nucleic acid concentration and purity were measured spectrophotometrically with a NanoDrop2000 (Thermo Fisher Scientific, USA) at 260 and 280 nm. The primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') were used to amplify the V4 hypervariable region of bacterial 16S rRNA genes. PCR was performed in a thermal cycler of which conditions as follows: initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 45 s, 50 °C for 45 s, 68 °C for 90 s, and a final extension step at 68 °C for 5 min. The purified PCR products were then mixed at equimolar ratios for sequencing on an Illumina MiSeq PE250 system (Illumina Corporation, USA) at Biomarker Technologies Co., Ltd, Beijing, China.

Microbiome bioinformatics was analyzed with QIIME 2 (2019.1) [27]. After raw sequence data were denoised and quality filtered with DADA2 (via q2-dada2) [28], 1,022,922 high-quality reads were obtained, with an average of 110,496 sequences per sample for the 9 bulk soil samples. After rarefaction, the taxonomy tables were generated using the plugin "q2-feature-classifier" [29] (Bokulich et al., 2018) with a pre-trained Naive Bayes classifier against the Silva 132 99% OTUs from 515F/806R region of sequences [30].

## 3. Results and discussion

### 3.1. Profile of soil pH, Eh, sulfate and nitrate in the flooding soil system

The pH of paddy soil increased mildly and was kept at a neutral condition in the 30 days. While the redox potential (Eh) of paddy soil decreased sharply in the first 5 days and become mildly decrease in the last days. These finding was constant with other studies, which confirmed that oxygen in soil solution is consumed fastly in the early period of flooding, and become stable because of aerobic microbial deaths, resulting in the reduction of Eh and in the increasing of pH simultaneously [31]. Eh of LN and HN treatments was relatively higher than that of control with a prolonged period of flooding (Fig. 1a). The results showed that the addition of nitrate could increase the mass of the oxidizing substance and alleviate the consumption of oxygen by use nitrate as an electric acceptor instead of oxygen, thus changed the redox state of flooding soils.

When Eh below 200 mV, microbes in turn use NO<sub>3</sub><sup>-</sup>, Mn<sup>4+</sup>, Fe<sup>3+</sup>, and SO<sub>4</sub><sup>2-</sup> as electron acceptors for reduction [31]. Nitrate concentration decreased from 1.06 to 0.79 mg g<sup>-1</sup> in HN treatment. While nitrate concentration in both CK and SCK is kept for almost 0.02 mg g<sup>-1</sup>, implied an inhibition of nitrification or denitrification process. The reason could be contributing to lower nitrate concentration and lower relative abundance of nitrification/denitrification associated microbes in CK treatment (results as shown in Fig. 2a). Different from nitrate, sulfate concentration decreased from 0.035 to 0.029 and 0.034 to 0.028 mg g<sup>-1</sup> in CK and SCK, respectively (results as shown in Fig. 2b). The addition of nitrate decreased SO<sub>4</sub><sup>2-</sup>, and the decreasing effect increased with nitrate dosage. Higher pH and lower Eh of CK and SCK induced release of SO<sub>4</sub><sup>2-</sup> from solid phase of paddy soils [32]. Even if nitrate competed with SO<sub>4</sub><sup>2-</sup> as electron acceptor, SO<sub>4</sub><sup>2-</sup> in CK & SCK was always higher than HN. There are researches pointed that the reduction of SO<sub>4</sub><sup>2-</sup> will influence the environmental behavior of As [33]. Sulfur derived from SO<sub>4</sub><sup>2-</sup> reduction could reduce Fe(III) and As(V) and promote As dissolution [34], while As could be immobilized as arsenic sulfide or FeS minerals [35,36]. It could be found that reduction of NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> occurred simultaneously with dissimilatory iron reduction, which further led to the reduction of arsenic.

### 3.2. Effect of nitrate addition on As(III) and dissimilatory iron reduction

Fig. 3 showed Fe(II) and As(III) concentrations in paddy soils of different treatments. In general, As(III) concentration was positive

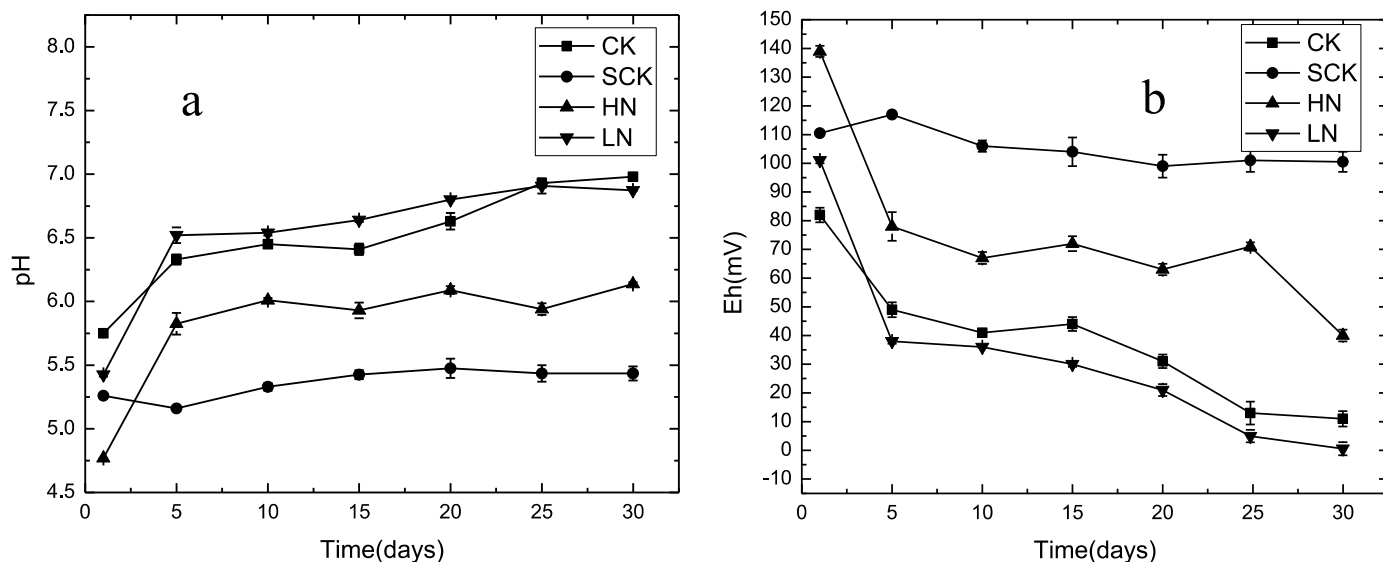


Fig. 1. pH(a) and Eh(b) change along with incubation time.

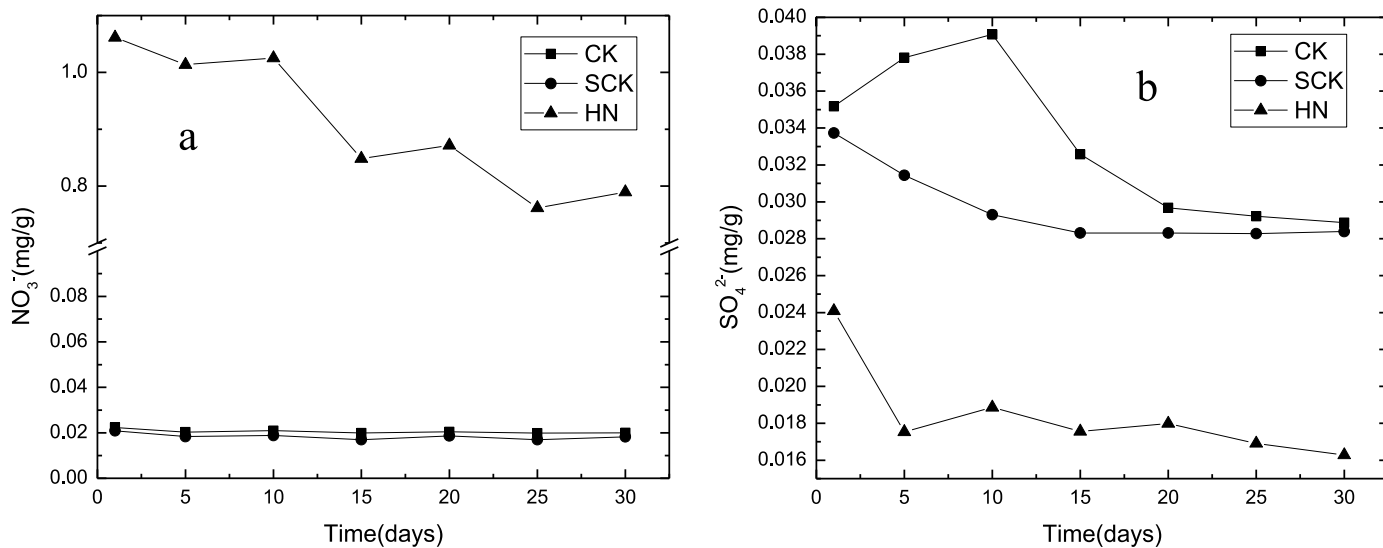


Fig. 2.  $\text{NO}_3^-$  and  $\text{SO}_4^{2-}$  concentration in paddy soils along with incubation time.

relative to Fe(II). It was found that Fe(II) increased quickly in the first 15 days for CK and 10 days for LN&HN treatments, while As(III) shows an opposite trend, As(III) concentration decreased in the first 15 days for CK and 10 days for LN&HN treatments. Considering Eh and pH behavior, oxygen that remained in porewater was consumed rapidly by microbes in the earlier stage of flooding, resulting in the form of an anaerobic or anoxic environment [37]. Even if Fe(III) dissolution was favored by this situation, As(III) concentration still decreased because of oxidization by some oxidizers such as MnOx [38].

The dissimilatory iron reduction could be strongly inhibited by the addition of nitrate. Compare  $\text{Fe}^{2+}$  concentration of HN&LN treatments and control, it can be found that  $\text{Fe}^{2+}$  in nitrate amendments are much less than control, especially for HN,  $\text{Fe}^{2+}$  concentration was 62.58% lower than control. The dissimilatory iron reduction was significantly depressed by the addition of nitrate. The results were slightly different from other studies, of which  $\text{Fe}^{2+}$  was almost disappeared in porewater. The reason is that they only detected  $\text{Fe}^{2+}$  in porewater of paddy soils while  $\text{Fe}^{2+}$  in paddy soils were extracted in our research. Both results indicated excellent immobile efficiency of nitrate for As, and the efficiency increased with the dosage of nitrate.

Fig. 3b showed As(III) concentration in the paddy soil system along with time. The results showed that As(III) concentration decreased from 319.85 to 249.25, 236.88 to 206.59, and 212.80 to 172.87  $\text{mg Kg}^{-1}$  in CK, LN, and HN treatments in the first 10 days, respectively. With the consumption of oxidizers, As(III) concentration began to increase after 10 days for LN&HN treatments and 15 days for CK treatment. The delay of CK treatment could be explained by more As(III) in CK, and an equal mass of oxidizing substances in CK, LN, and HN treatments. Due to the depletion of oxidizing substances, the arsenic oxidation process was basically completed and the reduction process started. The content of As(III) increased slowly and basically reached equilibrium at 25d. Final As(III) concentrations were 284.67, 223.87, and 190.70  $\text{mg Kg}^{-1}$  for CK, LN, and HN treatments, respectively. At this time, the dissimilatory iron reduction also basically reached an equilibrium state.

### 3.3. Microbial community structure change during the flooding period

The relative abundance of 15 dominant microbial families accounted for 70.60-78.79%, 57.12-64.21%, and 44.95-76.82% of total abundance for control, HN, and LN treatments, respectively (results as shown in

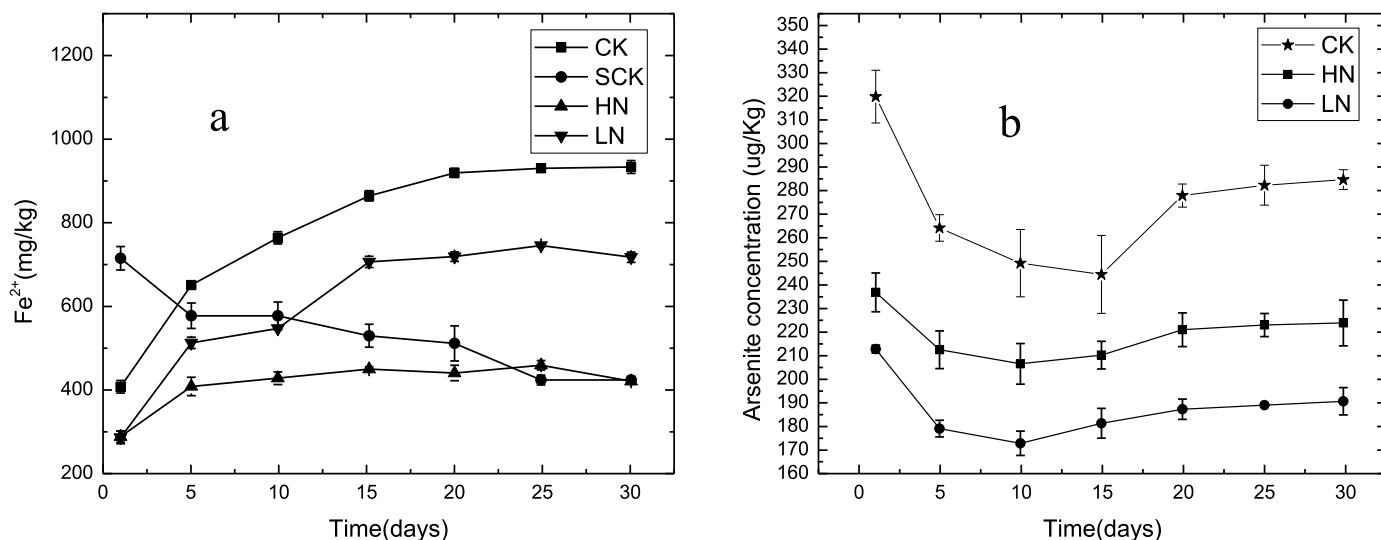


Fig. 3. Fe<sup>2+</sup>(a) and As(III)(b) concentration in paddy soils along with incubation time.

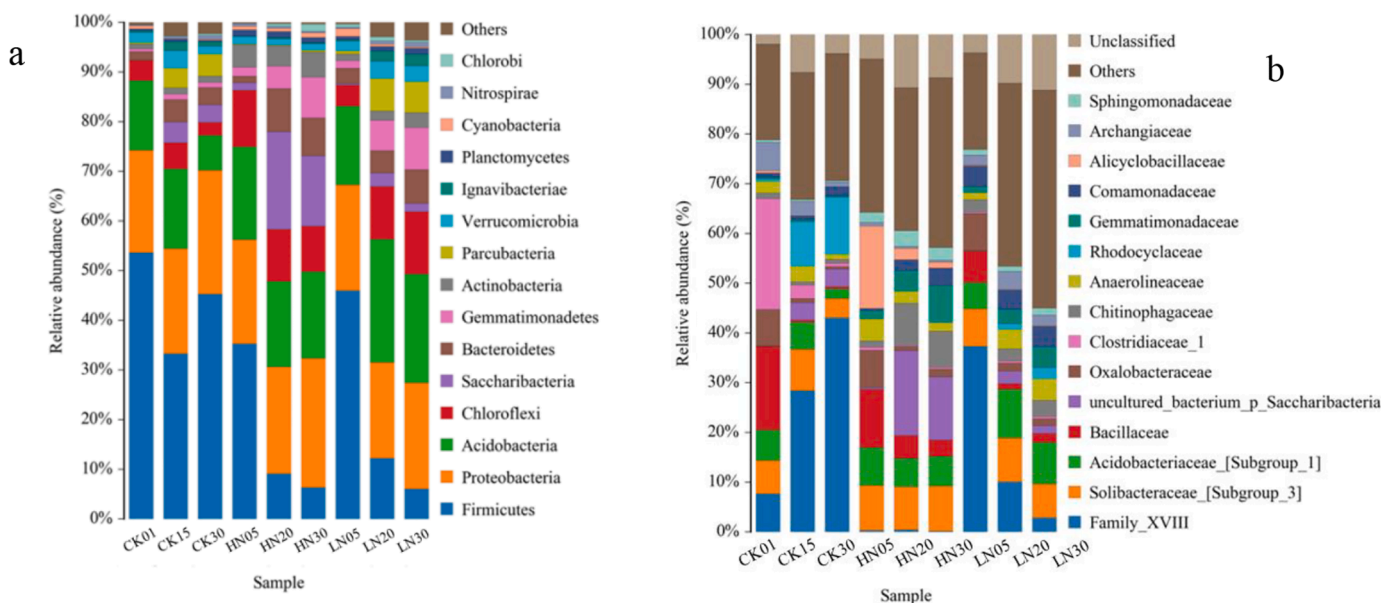


Fig. 4. Microbial community structure in Phylum(a) and Family(b) levels (marked number indicates the sampling time).

Fig. 4). At the Class level, the relative abundance of *Clostridia* increased from 34.76 % to 44.67% along with incubation time. Which was contrary to the trend of HN and LN treatments: relative abundance of *Clostridia* decreased from 2.34% and 39.24% to 1.20% and 3.92%, respectively. These results showed that the addition of nitrate strongly inhibited *Clostridia*, and the inhibition efficiency increased with nitrate dosage. On the contrary, the addition of nitrate promotes *Sphingobacteriia*, promotion is efficiently increased with dosage. Relative abundance of *Deltaproteobacteria* and *Parcubacteria* promoted by the low dosage of nitrate but inhibited by high dosage of nitrate. Relative abundance of *Bacilli* decreased from 18.15% to 0.45% in CK treatment, the addition of nitrate alleviate the decrease of *Bacilli*, even if it decreased along with incubation time. The influence of flooding and nitrate on *Betaproteobacteria* was insignificant.

With the decomposition of OC by the microorganisms, Eh decreased along with incubation time, this phenomenon was consistent with previous studies [39]. pH of every treatment increased with time, which was associated with reduction of NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> [40]. Along with

consumption of oxygen, strictly aerobic or facultative anaerobic bacteria such as *Clostridiaceae\_1* belonging to *Clostridiales* within *Firmicute* and *Bacillaceae* belonging to *Bacillales* within *Bacilli* were strongly depressed, especially for *Clostridiaceae\_1*, which decreased significantly in CK treatment (0.68%) and almost disappeared in HN and LN treatments (0.36% and 0.45%, respectively), indicated sharp inhibition of flooding on these bacteria.

*Firmicute* is the dominant phylum in CK treatment and increased along with incubation time, reached 45.24% after 30d incubation. *Family\_XVIII* belonging to *Clostridiales* within *Firmicute* was promoted most significantly, increased from 7.62% to 43.04% in CK treatment. On the contrary, nitrate inhibition the growth of *Family\_XIII*, the relative abundance decreased from 0.28% to 0.14% and 37.27% to 2.83% in HN and LN treatments, respectively. *Clostridiales Family\_XVIII* is a group of uncultured anaerobic bacteria whose growth is favored under oxygen-free conditions [41]. The ecological role of *Clostridiales Family\_XVIII* is not clear, while previous research indicates its relationship with denitrification and the iron-reducing process [42]. Thus, the addition of



nitrate inhibited some microbes such as *Clostridiales Family XVIII*, resulting in the promotion of denitrification and inhibition of the iron-reducing process.

Additionally, nitrate treatments promoted some microbes such as *Chitinophagaceae*, *Gemmatimonadaceae*, and *uncultured bacterium p Saccharibacteria*, the relative abundance of these microbes increased from 1.26% to 7.25%, 1.60 to 7.42% and 0.38% to 12.72% respectively for HN treatment, and increased from 2.46% to 3.22%, 1.11% to 4.17% and 0.11% to 1.60% for LN treatment. *Chitinophagaceae* and *Gemmatimonadaceae* had been proved to be promoted in the ammonia-nitrogen conditions, of which ammonia-nitrogen would be transformed into organic nitrogen [43]. While *uncultured bacterium p Saccharibacteria* were capable of degradation of organic nitrogen [44].

#### 4. Conclusion

The results of our research revealed the potential of nitrate amendment for immobilizing arsenic in flooded paddy soil efficiently. By reducing the extent of Eh decreasing, replacing Fe(III) as an electron acceptor, and regulating the change of corresponding microbial community structure change, the addition of nitrate inhibited dissimilatory iron reduction successfully, resulting in an impressive reduction of As (III) in submerged paddy soils. Microbes played a critical role in driving the environmental behavior of arsenic in flooded paddy soils, while nitrate is the driving factor for microbial community structure and associated ecological functions. During incubation of nitrate treatments, strictly aerobic or facultative anaerobic bacteria were strongly depressed; microbes associated with denitrification and iron-reducing process promoted by CK but depressed by nitrate; microbes take part in transform ammonia-nitrogen into organic nitrogen or degrade organic nitrogen were promoted by nitrate treatments. By link the geochemical process of  $\text{NO}_3^-$  with dissimilatory iron reduction, the source of arsenic release was blocked. On the other hand, nitrate-nitrogen is a type of fertilizer for rice, which means that nitrate can be used not only as a fertilizer but also as a stabilizer for As contaminated soils. This agronomy strategy is of particular importance for food security in most developing Asian countries which are the most important contributors to global rice production.

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Availability of data and materials

The data that support the findings of this research are available from the authors upon reasonable request.

#### Funding

This work is supported by the National Key R&D Program of China (No. 2019YFC1805305), the Key R&D Program of Guangdong Province, China (No.2019B110207001, No. 2020B1111350002), International Science and Technology Cooperation Projects of Guangdong Province, China (No. 2021A0505030045), the Special Basic Research Fund for Central Public Research Institutes of China (No. PM-zx097-202104-105) and Natural Science Foundation of Guangdong Province, China (No. 2019A1515012131).

#### Authors' contributions

All authors have read and agreed to the published version of the

manuscript.

#### CRediT authorship contribution statement

**Zhiliang Chen:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing. **Lihang An:** Data curation, Software, Writing – review & editing. **Hang Wei:** Conceptualization, Project administration, Software, Writing – original draft, Writing – review & editing. **Jianqiang Zhang:** Data curation. **Qi Zou:** Methodology. **Mengqiang Sun:** Methodology. **Ling Huang:** Writing – original draft. **Minchao Liu:** Funding acquisition, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no competing interests.

#### Acknowledgments

We would like to thank the anonymous reviewers and the editor.

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