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Iron oxidation coupled with nitrate reduction affects the acetate-assimilating microbial community structure elucidated by stable isotope probing in flooded paddy soil

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ABSTRACT

Acetate is an abundant carbon source that can trigger microbial redox processes in anoxic environments, thereby affecting the microbial community structure, function, and associated element cycling. Here, we investigated the acetate-assimilating microbial community, especially the key microorganisms involved in nitrate-dependent Fe (II) oxidation that are closely associated with soil redox processes in flooded soil. In the present study, DNA-stable isotope probing (DNA-SIP) with labeled acetate (^{13}C) as a carbon source was applied to examine the acetate-assimilating communities associated with nitrate-dependent Fe(II) oxidation. The results showed that NO_3^- was rapidly reduced in the treatments with NO_3^- and NO_3^- + Fe(II). Fe(II) oxidation occurred quickly only in the presence of NO_3^-. In the treatments with acetate only, the predominant ^{13}C-labeled genera such as *Geobacter*, *Azospira*, *Azospirillum*, *Ideonella*, and *Desulfovibrionia* were probably involved in acetate oxidation coupled with the redox processes of NO_3^-, Fe(III) and SO_4^{2-} reduction, which are the most important electron acceptors in flooded soils. The addition of NO_3^- and Fe(II) significantly affected the acetate-assimilating microbial community from the original soil. The enriched genera in ^{13}C heavy fractions were associated with *Pseudogulbenkiania*, *Azospira*, *Zoogloea*, *Azoarcus*, and *Bdellovibrio* dominated in the treatments with NO_3^- and Fe(II). In the treatments with NO_3^- only, *Zoogloea*, *Azospira*, *Azoarcus*, and *Geothrix* were the dominant genera. Given the different genera with enrichments in ^{13}C-heavy fractions in different treatments, *Zoogloea* and *Pseudogulbenkiania* were identified as key microorganisms associated with NO_3^- reduction and nitrate-dependent Fe(II) oxidation, respectively. These findings suggest the importance of *Zoogloea* and *Pseudogulbenkiania* for C, Fe, and N biogeochemistry and indicate that Fe and N cycling have a great impact on soil biogeochemistry processes.

1. Introduction

In flooded paddy soils, anaerobic microbial processes are major drivers of the cycling of organic carbon coupled with the reduction of inorganic electron acceptors (Hori et al., 2007; Xu et al., 2019). Acceptors such as nitrate (NO_3^-), Fe(III), sulfate (SO_4^{2-}) and carbon dioxide (CO_2), can act as the exogenous electron acceptors for the indigenous microorganisms in flooded soils (Yao et al., 1999). According to the thermodynamic theory, these electron acceptors in paddy soil are reduced sequentially under anoxic conditions: NO_3^- first, followed by Fe (III) oxides, SO_4^{2-}, and CO_2 (Yao et al., 1999; Hori et al., 2010). Nitrate reduction is predicted to be one of the most important electron sinks in many paddy soils, and the microorganisms involved in NO_3^- reduction processes are typical NO_3^--reducing bacteria or nitrate-dependent Fe (II)-oxidizing bacteria (Yoshida et al., 2012; Li et al., 2016). In a previous study that investigated a red soil in southern China, over 10 mM Fe (II) could be detected, and Fe(II) was soluble and more bioavailable for microbial Fe(II) oxidation under flooded conditions (Li et al., 2018). The

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coexistence of NO_3^- and Fe(II) creates an ideal environment for nitrate-dependent Fe(II) oxidation processes by functional microorganisms (Ishii et al., 2016; Li et al., 2016). This microbial reaction plays a critical role in the biogeochemical cycling of carbon, iron, and nitrogen.

Various bacteria have been described as denitrifying bacteria that can also oxidize Fe(II) coupled to NO_3^- reduction (Ratering and Schnell, 2001). Most nitrate-dependent Fe(II)-oxidizing bacteria isolated to date belong to the Proteobacteria and Actinobacteria phyla, and are found in a wide range of habitats, including sediment, freshwater, estuarine, and marine habitats (Ratering and Schnell, 2001). Among these microorganisms, a minority do not require organic substances for energy generation. These microorganisms can use Fe(II) as the sole electron door and fix CO_2 to produce biomass (Bryce et al., 2018). In contrast, most other nitrate-dependent Fe(II)-oxidizing bacteria require additional organic carbon for growth to continually oxidize Fe(II), and NO_3^- can be further reduced stepwise to NO_2^-, NO, N_2O and N_2 in a sequential reaction (Li et al., 2016; Zhang et al., 2020). Previous reports have confirmed that a variety of organic carbon sources can be used to support the growth of nitrate-dependent Fe(II)-oxidizing bacteria (Tominski et al., 2018). For example, acetate stimulated the growth of the heterotrophic nitrate-dependent Fe(II) oxidizers *Bradyrhizobium*, *Comamonas badia*, *Parvibaculum lavamentivorans*, and *Rhodanobacter thiooxidans* in an Fe(II)-oxidizing, nitrate-reducing enrichment culture (Blöthe and Roden, 2009; Tominski et al., 2018). Li et al. (2016) found that *Azospira*, *Zoogloea*, and *Dechloromonas* were associated with acetate assimilation during Fe(II) oxidation in the presence of NO_3^-. Nevertheless, owing to the challenge in isolating and culturing these putative nitrate-dependent Fe(II) oxidizers, not much know is about the identification of carbon-assimilating microorganisms capable of Fe(II) oxidation and NO_3^- reduction, thereby overlooking the connection between organic carbon assimilation and the ecological functions of these heterotrophic microorganisms.

A direct way of linking identify of microorganisms to a particular set of metabolic processes in the natural environments is stable isotope probing (SIP) (Dumont and Murrell, 2005; Hori et al., 2010). SIP incubation combined with high throughput sequencing can provide direct evidence of the impact of functional microorganisms on element assimilation without laboratory culturing (Dumont and Murrell, 2005). During the growth of functional microorganisms with a labeled (^{13}C) carbon source, the produced ^{13}C-DNA can be separated and further analyzed taxonomically. Osaka et al. (2006) used ^{13}C-acetate as the carbon source to clarify the growth of microbial populations under nitrate-reducing conditions in activated sludge. Furthermore, these key microorganisms dominated in the microbial community and were isotopically labeled with ^{13}C-acetate oxidation coupled with pentachlorophenol and manganese reduction (Xu et al., 2019). Among these studies, acetate is an abundant carbon source that can trigger microbial redox processes to influence the geochemical cycle of elements in anoxic environments (Liu et al., 2019; Kappler et al., 2021). However, as most studies have concentrated on the redox processes, the effect of acetate on the diversity of functional microorganisms has been neglected. Acetate oxidation has so far not been directly linked to specific populations in flooded paddy soil.

In the present study, to better understand the effect of carbon source on the redox processes (NO_3^- reduction and Fe(II) oxidation) and the diversity of heterotrophic nitrate-dependent Fe(II) oxidizers in flooded paddy soils, ^{13}C-labeled acetate, one of the most important substances for microbial respiration under flooded paddy soils, was used to trace specific biogeochemical processes and key associated microorganisms involved in nitrate-dependent Fe(II) oxidation. DNA-SIP combined with 16S rRNA gene amplicon sequencing was applied to identify the composition of acetate-assimilating microorganisms and heterotrophic nitrate-dependent Fe(II) oxidizers. The obtained results provide important insight into the distribution and diversity of acetate-utilizing nitrate-dependent Fe(II) oxidizers in flooded paddy soils. Moreover, regulating the growth of nitrate-dependent Fe(II) oxidizers may assist in the remediation of nitrate-heavy metal complex contaminated soils because various Fe(III) oxides formed by Fe(II) oxidizers could co-precipitate or adsorb soluble heavy metals.

2. Materials and methods

2.1. Sample collection

Paddy soil was collected from Zhangshi Village, Shaoguan City (25°33′26″N, 113°29′42″E), Guangdong Province, China in September 2021. This city has a typical humid subtropical climate in south China. The annual mean temperature is 21 °C. The typical cropping system in this area is double-cropping rice (two rice seasons). The soil sample was obtained from the drainage period (at depth of 0–20 cm) of the rice growth cycle. The soil samples were mixed using the quartation method and stored in polyvinylchloride bottles. Then, the samples were transferred immediately to the laboratory and stored at 4 °C in a refrigerator for incubation. The samples used for DNA extraction and analysis were stored at −80 °C until use. The physicochemical properties of the soil are shown in Table S1.

2.2. Incubation setup

The pre-incubation of soil slurry was performed by mixing the sterile water and soil for 1 week at a ratio of 1:1 to activate the indigenous microorganisms under dark conditions (Hori et al., 2007). After pre-incubation, aliquots of soil slurry were transferred into 50 ml sterile serum bottles containing 30 ml of piperazine-1,4-bisethanesulfonic acid buffer (PIPES, 20 mM, pH 7.0) in the dark at a constant temperature of 25 ± 1 °C. These bottles were flushed with nitrogen for 30 min to replace the headspace and then closed with butyl rubber stoppers and aluminum crimp seals. Different treatments were prepared in triplicates: (i) sterile soil amended with NO_3^- and Fe(II); (ii) soil with only acetate, (iii) soil with acetate and NO_3^-; (iv) soil with acetate and Fe(II), and (v) soil with acetate, NO_3^-, and Fe(II). The soil used for sterile controls was sterilized by irradiation at 50 kGy. The final concentrations of acetate, NO_3^-, and Fe(II) were 5 mM, 10 mM, and 5 mM according to previous reports, respectively (Klueglein et al., 2014; Li et al., 2018). To investigate the effects of NO_3^- reduction and Fe(II) oxidation on the acetate-assimilating bacteria, DNA-SIP was performed, and incubations were amended with ^{13}C-labeled or unlabeled acetate. The treatments and the concentrations of appropriate substrate were the same as in the above five treatments. All incubations were sampled destructively for Fe(II), NO_3^-, and NO_2^- measurements at 0, 0.5, 1, 1.5, 2, 3, and 4 days. The concentrations of ^{13}C-biomass, $^{13}CH_4$, and $^{13}CO_2$ were determined at the end of the incubation.

2.3. Analytical methods

The dissolved Fe(III) and Fe(II) concentrations were determined as described previously (Tong et al., 2021). During incubation, 1 ml of culture slurry was centrifuged at 10,000 *g*, and the supernatant was passed through a 0.22 μm filter. After acidification with 50 μl of concentrated HCl, the Fe(II) concentration was determined with the 1, 10-phenanthroline colorimetric method. The acidified solution were reduced using 0.25 mol/L hydroxylamine, mixed with 1,10-phenanthroline, and subsequently analyzed to determine the total Fe concentration. The difference between the values of the dissolved Fe(II) and the total Fe concentrations represented dissolved Fe(III). The NO_3^-, NO_2^-, and SO_4^{2-} concentrations in the slurry after filtration were measured using ion chromatography equipped with an IonPac AS14A anion exchange column (ICS-90, Dionex, CA, USA). The rates of microbial Fe(II) oxidation and nitrate reduction were calculated by the pseudo-first-order kinetic model as described previously (Li et al., 2018). The methods used to determine CH_4 and CO_2 concentrations in the headspace and the ratio of $^{13}C/^{12}C$ in CH_4 and CO_2 were described previously (Tong et al., 2015).

The method used for microbial biomass C determination in the ^{13}C-labeled acetate treatment was described by Tong et al. (2018). The supernatant was removed from the slurry via centrifugation, and the microbial biomass C of the residual was measured using the chloroform fumigation extraction method (Wu et al., 1990). The extracts were freeze-dried for total organic carbon (TOC) measurement with a TOC analyzer (TOC 5000A with SSM 5000A module, Shimadzu, Kyoto, Japan), and the $\delta^{13}C$ was measured using an isotope ratio mass spectrometry system (IRMS, Thermo Scientific, Bremen, Germany). The method used for the calculation of the ^{13}C microbial biomass was reported previously (Khan et al., 2008).

2.4. DNA extraction, SIP gradient fractionation, and qPCR

Genomic DNA was extracted from approximately 0.3 g soil from both the ^{12}C- and ^{13}C-labeled incubations with the PowerSoil™ DNA isolation kit (Qiagen, Hilden, Germany) at 0, 2, 4, and 10 days. The DNA was quantified with a Qubit 2.0 Fluorometer DNA (Invitrogen, Carlsbad, CA, USA). Approximately 10 μg of DNA at days 2, 4, and 10 was placed into Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 ml, Beckman Coulter, CA, USA) together with Tris-EDTA-CsCl solution (TE, pH 8.0) as described previously (Sun et al., 2021). Before sealing, the buoyant densities (BDs) in the tubes were measured with a model AR200 digital refractometer (Reichert Inc., Depew, NY, USA). DNA was centrifuged at 178,000 *g* for 48 h at 20 °C in a Stepsaver 70 V6 vertical titanium rotor (Thermo Scientific, CA, USA). Gradients of DNA were removed using a Beckman fraction recovery system (Beckman Coulter, USA). The separated DNA was divided into heavy fractions (^{13}C-DNA) and light fractions (^{12}C-DNA), which were used in the results and discussion section. The heavy and light fractions were precipitated with glycogen (dissolved in 30% ethanol) and subsequently eluted with 40 μl TE buffer (Zhang et al., 2021). The bacterial 16S rRNA genes in every fraction were determined via qPCR on a MyiQ™ 2 Optics Module (BIO-RAD, CA, USA) with the primers 338F and 518R using the following temperature and cycling: 3 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C (Ding et al., 2020). All samples were run in three technical repetitions. The qPCR calibration curves were generated with serial dilutions (ranging from 1×10^2 to 1×10^9 copies/μl) of plasmids containing the cloned target sequences. The plasmid DNA concentration was quantified by Qubit 2.0 Fluorometer (Invitrogen, NY, USA), and the corresponding gene copy number was calculated relatively to the plasmid size, insert lengths and Avogadro number (Whelan et al., 2003).

2.5. Illumina MiSeq sequencing and community analysis

For community analysis, genomic DNA from incubation at 0, 2 and 4 days was sequenced using an Illumina Miseq platform with the 16S rRNA gene primers 338F and 806R (Tong et al., 2018). Based on the results of the qPCR of the bacterial 16S rRNA genes, several representatives of heavy and light fractions that contained the highest 16S rRNA gene abundance were sequenced using the primers 338F and 806R. The PCR amplicons were purified using the UltraClean PCR clean-up kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) and then quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). A single composite sample was produced that contained barcoded PCR product, normalized in equimolar amounts to produce equivalent sequencing depth from all samples. The 12-bp barcode was used to assign individual sequences to samples. The 16S rRNA gene amplicons were submitted to Magigene Biotechnology (Guangzhou, China) for Miseq Illumina high-throughput sequencing (Illumina Miseq platform, PE 250). The chimeric and low-quality sequences were identified and removed using Mothur programs (Schloss et al., 2009). All qualified reads were grouped into operational taxonomic units (OTUs) at the 97% sequence similarity level using UCLUST software (http://www.drive5.com/usearch/) (Edgar, 2010). The taxonomic classification of each phylotype was determined using the Ribosomal Database Project (RDP) at the 80% threshold. The relative abundance (%) of individual taxa within each community was estimated by comparing the number of sequences assigned to a specific taxon compared with the number of total sequences obtained for that sample. The microbial communities from the incubation cultures of different treatments were analyzed by principal coordinate analysis (PCoA) with pairwise unweighted UniFrac distance (Lozupone and Knight, 2005). The raw sequences were deposited in the SRA database in the National Center for Biotechnology Information (NCBI, Accession No. SRP5408992).

3. Results

3.1. Activity of NO_3^- reduction and nitrate-dependent Fe(II) oxidization

In the sterile control, no Fe(II) oxidation or NO_3^- reduction was observed, suggesting no chemical interaction among the electron donor, Fe(II), and NO_3^- (Fig. 1). Following 4 days of incubation, complete Fe(II) oxidation occurred in the treatments with Fe(II) and NO_3^- at a rate of 0.127 mM h^{-1}, and the Fe(III) concentration reached 2.87 ± 0.19 mM (Fig. 1A). In the treatments with Fe(II) only, the concentration of dissolved Fe(II) was slightly different from the sterile controls, indicating that only a small amount of Fe(II) was oxidized in the absence of NO_3^-. This is consistent with concentration of Fe(III) in the treatments with Fe (II) only (Fig. 1A). No Fe(III) was detected in the sterile controls, suggesting that no microbial Fe(II) oxidation occurred (Fig. 1A). In the sterile controls, the decrease in Fe(II) concentration was due to the adsorption of soil particles. Nitrate was rapidly reduced within 2 days, and then the reduction rate slowed in the treatments with NO_3^- (Fig. 1B). In the presence of Fe(II), NO_3^- reduction slowed with 64% of the NO_3^- reduced after incubation for 4 days. The rates of NO_3^- reduction were 0.167 and 0.093 mM h^{-1} in the treatments with NO_3^- and NO_3^- + Fe(II), respectively. These results suggested that NO_3^- was reduced by indigenous microorganisms and that microbial NO_3^- reduction was inhibited in the presence of Fe(II). During NO_3^- reduction, the NO_2^- concentrations increased at the beginning of the incubation period, reached a maximum value, and then decreased over time (Fig. 1B). Neither NO_3^- reduction nor NO_2^- production was observed in the sterile controls.

During acetate consumption, the labeled C was transformed into biomass or other metabolites. The assimilation of ^{13}C-acetate into biomass after incubation for 4 days is shown in Fig. S1. The results showed that the yield of ^{13}C-biomass did not differ among different treatments. By comparison, the emission of $^{13}CO_2$ and $^{13}CH_4$ in the treatments amended only with acetate was significantly higher than in the treatments with NO_3^- and NO_3^- + Fe(II) ($P < 0.05$, Fig. S1). The different ^{13}C percentages among the three treatments suggested that the amended NO_3^- and/or Fe(II) affected the microbial community structure and function, possibly by inhibited bacterial anaerobic respiration using acetate. The percentages of labeled acetate recovered in the total gases ($^{13}CO_2$ and $^{13}CH_4$) did not differ between the treatments with NO_3^- and NO_3^- + Fe(II).

3.2. Microbial composition in the course of nitrate-dependent Fe(II) oxidization

Significant differences in the microbial communities among the original soil and different treatments were observed using high-throughput sequencing ($P < 0.05$, Fig. S2). The results of PCoA showed that the microbial nitrate reduction or nitrate-dependent Fe(II) oxidation affected the microbial composition of acetate-respiring cultures (Fig. S2). Because no significant microbial Fe(II) oxidation occurred in the treatments with acetate and Fe(II), the microbial communities in these treatments were not analyzed in the present study. After filtering out low quality sequences, the obtained sequences ranged from 73,209 to 83,128. In the treatments with only acetate, the dominant genera were *Zoogloea*, *Azospira*, *Geobacter*, *Anaerolinea*, *Bacillus*, *Clostridium*, *Ideonella*, *Anaeromyxobacter*, and *Hydrogenispora* after

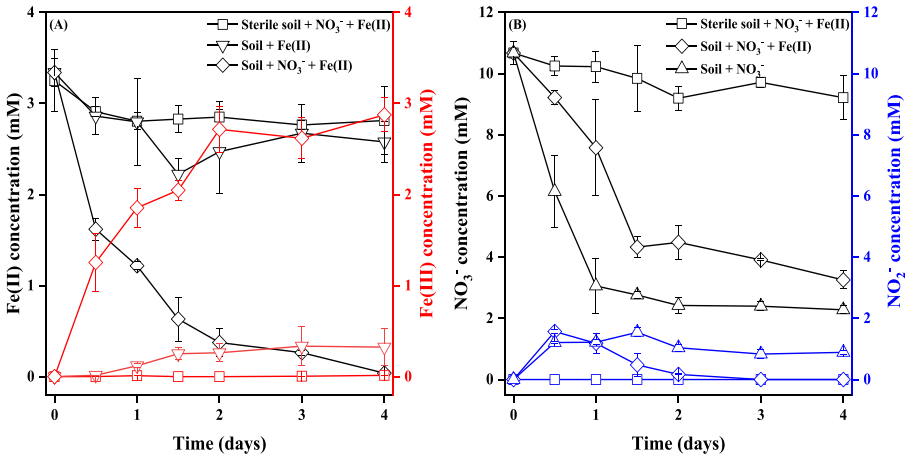

Fig. 1. Concentrations of dissolved Fe(II) (black line) and Fe(III) (red line) (A), and NO_3^- (black line) and NO_2^- (blue line) (B) over time in different treatments with acetate. The sterile control contained NO_3^- and Fe(II).

incubation for 4 days (Fig. 2). Compared with the treatments with only acetate, the relative abundances of *Zoogloea* and *Azospira* significantly increased from 2.1% to 42.8% and 0.8%–7.1%, respectively, while other dominant genera decreased in the treatment with acetate and NO_3^-. In the treatments with acetate, NO_3^-, and Fe(II), *Pseudogulbenkiania* (30.2%), *Zoogloea* (5.8%), *Azoarcus* (5.5%), and *Bdellovibrio* (3.3%) became the dominant genera, accounting for nearly 50% of the relative abundance (Fig. 2). The different abundances of dominant microorganisms among these treatments indicated that the addition of NO_3^- and Fe(II) promoted the growth of special microorganisms, such as nitrate-reducing bacteria and nitrate-dependent Fe(II)-oxidizing bacteria.

3.3. Potential acetate-respiring microbial community indicated by SIP analysis

To better understand which microorganisms played a role in acetate assimilation, the genomic DNA of labeled and unlabeled acetate microcosms was ultra-centrifuged with a CsCl gradient and then separated into different fractions along a density gradient ranging from 1.660 to 1.780 g mL^{-1} (Fig. S3). The results of 16S rRNA gene copies showed that the maximum relative abundance shifted to heavier fractions from 1.71 g mL^{-1} in the ^{12}C-acetate treatments to 1.74 g mL^{-1} in the ^{13}C-acetate treatments (Fig. S3). The peak fraction shifting to heavy fractions from incubations with ^{13}C-acetate compared to that from the unlabeled acetate suggested the incorporation of ^{13}C-acetate by some functional microorganisms. Fractions around the highest relative abundances of the 16S rRNA gene from different treatments were selected for high-throughput sequencing. Further analysis of the representative gradient fractions from all treatments were used to compare their microbial community structures and decipher the influence of NO_3^- and Fe(II) on acetate-respiring microorganisms.

The most abundant bacteria from heavy gradient fractions in the treatments with ^{13}C-acetate only after incubation for 4 days are summarized in Fig. 3. Compared with the difference between ^{12}C and ^{13}C treatments, most of these potential ^{13}C-assimilating microorganisms belonged to the Proteobacteria phylum, including the genera *Geobacter*, *Azospira*, *Azospirillum*, *Ideonella*, *Dechloromonas*, *Pseudomonas*, and *Zoogloea*. There were shifts to heavier fractions in the incubations with ^{13}C-acetate for these dominant genera compared to the peak fraction in unlabeled DNA (Fig. S4). In addition, some members of *Acidobacter*, *Chloroflexota*, *Desulfovibrionia*, *Holophagae*, and *Myxococcota* were also enriched in ^{13}C-heavy fractions. Members affiliated with *Geobacter* dominated in ^{13}C-acetate-heavy fractions with an average relative abundance of 27.8%, followed by *Azospira* (16.2%) and *Anaeromyxobacter* (5.0%). Additionally, *Azospirillum*, *Ideonella*, and *Pseudomonas* were highly enriched in ^{13}C-acetate-heavy fractions and showed a

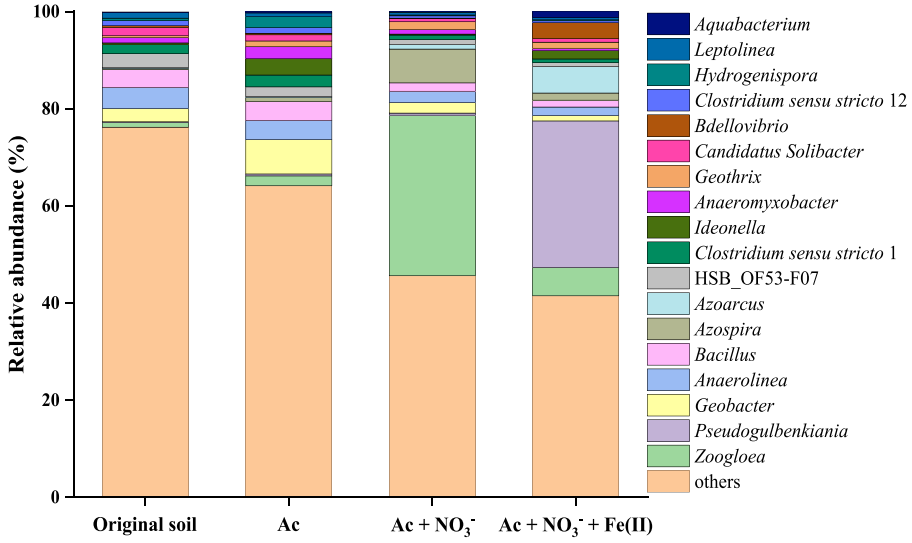

Fig. 2. Relative abundances of the 18 most abundant microbial genera in the original soil and incubation samples after incubation for 4 days. Ac represents acetate.

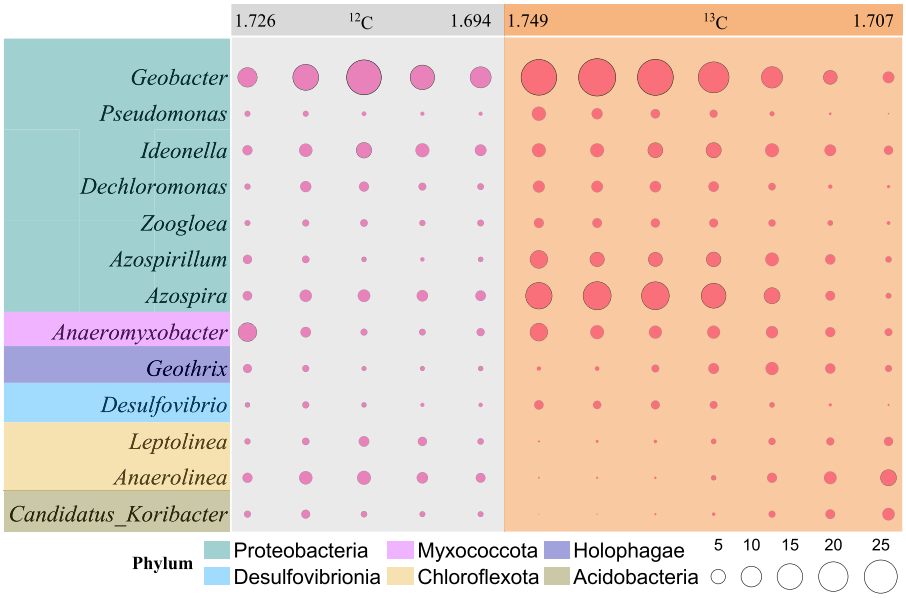

Fig. 3. Relative abundance of 16S rRNA genes across CsCl gradient heavy fractions from the treatments with ^{12}C- or ^{13}C-acetate-only in the original soil after incubation for 4 days. The buoyant densities decrease from left to right.

maximum relative abundance of approximately 5%.

3.4. Representative ^{13}C-assimilating microorganisms under NO_3^- reduction and Fe(II) oxidation

The effects of NO_3^- and Fe(II) on ^{13}C-acetate assimilating microorganisms were investigated via PCoA in the ^{13}C-heavy fractions from different treatments (Fig. 4). The community compositions in the representative heavy fractions were significantly different among the treatments with acetate only, NO_3^- and NO_3^- + Fe(II), indicating that NO_3^- reduction and Fe(II) oxidation led to a difference in the communities that incorporated ^{13}C ($P < 0.01$). Additionally, the microbial compositions of the heavy fractions in ^{13}C–NO_3^- and NO_3^- + Fe(II) treatments were different, indicating that nitrate reduction coupled with Fe(II) oxidation significantly altered the ^{13}C-assimilating microbial communities ($P < 0.01$). The positive or negative response in microbial composition change observed under NO_3^- or NO_3^- + Fe(II) stress is shown in Fig. 5. If the relative abundance of a species increases during incubation with NO_3^- or NO_3^- + Fe(II) compared with the treatment with acetate only, we refer to this species as a positive responder. Conversely, this species is considered as a negative responder when the relative abundance decreases. The relative abundance of *Geobacter*, the dominant genus in the ^{13}C-acetate-only treatments, significantly decreased from 31.8% to 2.0% and 1.7% in the ^{13}C–NO_3^- and NO_3^- + Fe(II)

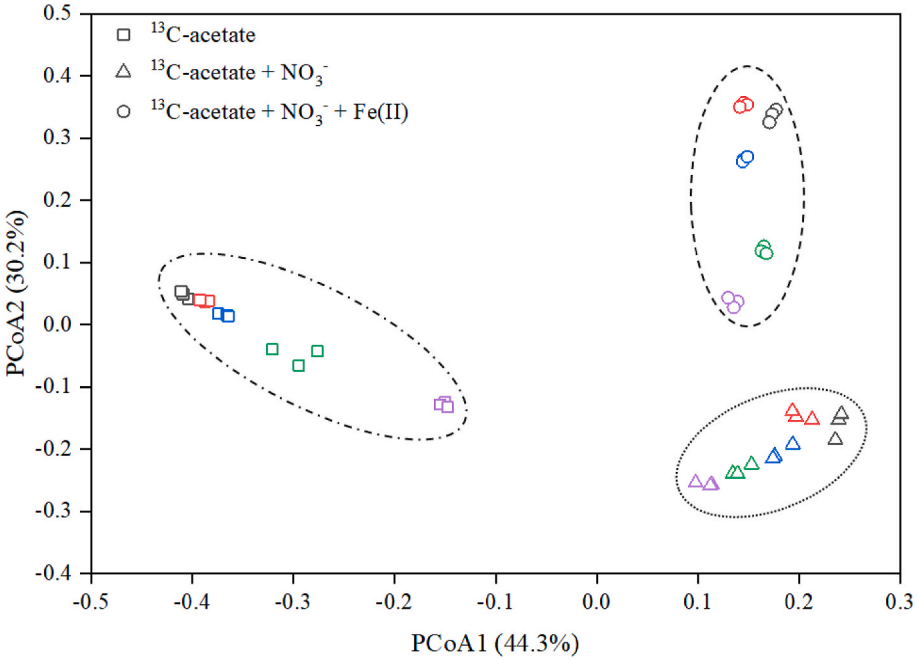

Fig. 4. PCoA comparing the microbial composition in ^{13}C-heavy fractions under different treatments. Square, triangle, and circle indicate the treatments with ^{13}C-acetate, ^{13}C-acetate + NO_3^-, and ^{13}C-acetate + NO_3^- + Fe(II), respectively. Black, red, blue, green, and purple show the buoyant density from 1.749 to 1.754, 1.741–1.748, 1.735–1.740, 1.727–1.732, and 1.720–1.726 g mL^{-1}, respectively.

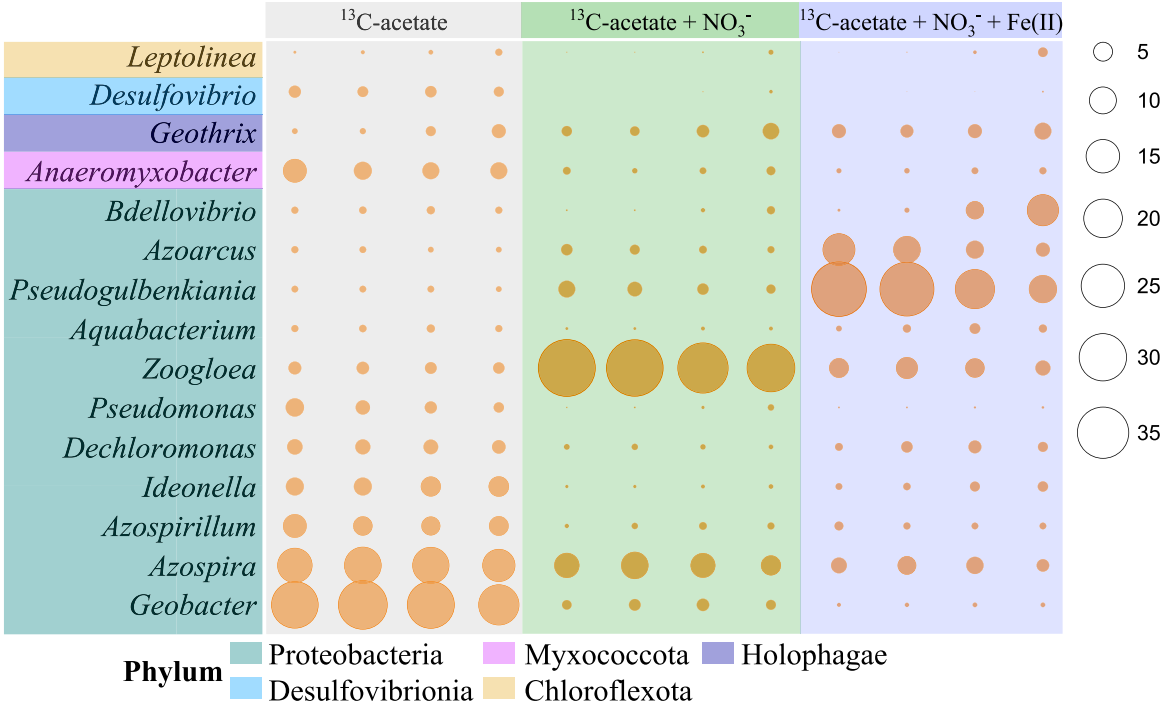

Fig. 5. Relative abundance of 16S rRNA genes across CsCl gradient fractions in ^{13}C-heavy fractions among the different ^{13}C treatments. The buoyant densities decrease from left to right.

treatments, respectively. Bacteria affiliated with *Zoogloea* increased from 2.1% to 42.7% in the treatments with NO_3^-, while *Pseudogulbenkiania* increased from 0.5% to 39.7% in the treatments with NO_3^- + Fe(II). In total, the most positive response under the NO_3^- treatments were affiliated with members of *Zoogloea*, *Azospira*, *Geobacter*, *Geothrix*, and *Azoarcus*, while the dominant genera were *Pseudogulbenkiania*, *Azospira*, *Zoogloea*, *Azoarcus*, *Geothrix*, and *Bdellovibrio* in the treatments with NO_3^- and Fe(II) (Fig. 5). The change in BD showed that these dominant microorganisms were enriched in the ^{13}C-heavy fractions but not in the ^{12}C-heavy fractions (Figs. S5 and S6). The positive genera were identified as NO_3^- reducers or nitrate-dependent Fe(II) oxidizers in the present study with SIP (Xu et al., 2019). The maximum relative abundances of these genera were enriched in and shifted to the ^{13}C-heavy fractions, suggesting that these microorganisms could assimilate ^{13}C into DNA via NO_3^- reduction or nitrate-dependent Fe(II) oxidation.

4. Discussion

Acetate, as one of the most important carbon sources in anoxic environments, is oxidized to CO_2 and assimilated by microorganisms carrying out anaerobic respiratory processes, such as NO_3^-, Fe(III), and SO_4^{2-} reduction (Chidthaisong and Conrad, 2000; Hori et al., 2010; Jiang et al., 2022). Therefore, acetate is an ideal carbon source to identify functional microorganisms involved in these anaerobic respiratory processes. Previous studies showed that labeled acetate with DNA- or RNA-SIP can be used to investigate acetate-dependent respiratory processes, such as pesticide degradation, Fe(III) reduction, and SO_4^{2-} reduction (Hori et al., 2010; Liu et al., 2018; Xu et al., 2019). In the present study, it was found that labeled acetate could be linked with NO_3^- reduction and nitrate-dependent Fe(II) oxidation.

4.1. ^{13}C-acetate assimilation linked to soil redox processes

In the present study, a variety of electron acceptors detected in the original soil (Table S1) could be used by anaerobically respiring microorganisms in flooded paddy soils when acetate was added as an electron donor, producing CO_2 and CH_4. At the end incubation with acetate only, the dissolved NO_3^- and SO_4^{2-} were not detected while the Fe (II) concentration increased to 8.54 ± 0.37 mM, confirming the occurrence of soil redox processes. Comparative SIP with ^{12}C- and ^{13}C-acetate permitted the detection of DNA labeled with ^{13}C-acetate assimilation. The high-throughput sequencing results from ^{13}C-heavy fractions showed that the dominant genera *Geobacter*, *Ideonella*, *Anaeromyxobacter*, *Pseudomonas*, *Azospira*, *Azospirillum*, and *Desulfovibrio* actively participated in ^{13}C assimilation in the ^{13}C-acetate treatments (Fig. 3 and S4). Members of *Geobacter* belong to the family *Geobacteraceae*, which contains the most common and comprehensively studied Fe(III)-reducing bacteria (Weber et al., 2006). Previous studies have shown that acetate can stimulate the rapid growth of *Geobacter* in association with the rapid reduction of Fe(III) oxides (Holmes et al., 2002; Risso et al., 2008). *Ideonella*, *Anaeromyxobacter*, and *Pseudomonas*, which had lower relative abundance in ^{13}C-heavy fractions, have also been reported as Fe(III) reducers (Ganne et al., 2017; Wang et al., 2020). *Azospira* and *Azospirillum*, both of which are known as NO_3^- reducers, have been observed in a variety of anoxic environments (Florio et al., 2019; Mikes et al., 2021). *Desulfovibrio* contains the most widely studied sulfate-reducing microorganisms, which can couple acetate oxidation to the reduction of SO_4^{2-} (Baffert et al., 2019). Therefore, these dominant genera in ^{13}C-heavy fractions likely participated in soil redox processes coupled with the oxidation of electron donors. According to the thermodynamic theory, oxidants in soil are reduced sequentially with NO_3^- first, followed by Fe(III) oxides, and SO_4^{2-} under anoxic conditions (Hori et al., 2010). This was consistent with the relative abundances of the dominant genera in heavy fractions, in which Fe(III) and NO_3^- reducers were the most abundant, followed by SO_4^{2-} reducers (Fig. 2). The difference in the relative abundance between Fe(III) and NO_3^- reducers may have been due to the variations in Fe(III) and NO_3^- content (Table S1).

4.2. Effect of nitrate and Fe(II) on microbial acetate oxidation

The soil redox processes in this study occurred in anoxic paddy soil,

in which acetate was oxidized into CO_2 and CH_4 (Xu et al., 2019). The ^{13}C results showed that acetate was mainly converted to $^{13}CO_2$ and $^{13}CH_4$, accounting for 40–60% of the carbon recovery (Fig. 2), which was consistent with other acetate-assimilating incubations with ^{13}C or ^{14}C acetate (Chidthaisong et al., 1999; Hori et al., 2007). Some of the unrecovered labeled C was incorporated into biomass as labeled DNA (^{13}C-DNA) (Fig. S3), whereas another fraction might have resulted from incomplete oxidation of ^{13}C-acetate during incubation (Chidthaisong and Conrad, 2000). In the present study, the supernatant containing ^{13}C-acetate was removed before measuring ^{13}C-biomss, which led to some ^{13}C loss. In the treatments with acetate only, the $^{13}CH_4$ production was significantly higher than the $^{13}CO_2$ production, and it was also higher than that of other treatments with NO_3^- and Fe(II) (Fig. S1). The possible reason was that the acetate only treatments enriched the *Geobacter* species, which could increase CH_4 production through direct interspecies electron transfer (McAnulty et al., 2017; Mei et al., 2018). A previous study showed that some microorganisms preferentially utilized acetate-derived metabolites or CO_2 as substrates to produce CH_4 (Demirel and Scherer, 2008). The addition of NO_3^- or NO_3^- + Fe(II) resulted in a decrease in gaseous products (CO_2 plus CH_4) compared with the treatments with acetate only. Nitrate reduction might have caused this by reducing microbial diversity and methanogen abundance (Yang et al., 2016). Moreove, NO_3^- and its denitrification products, such as NO_2^-, N_2O, and NO, have been previously reported to inhibit methanogenesis under anaerobic conditions (Chidthaisong and Conrad, 2000; Okutman Tas and Pavlostathis, 2008). Among these products, NO is particularly toxic due to its reactivity with heme and nonheme iron-containing proteins (Ye et al., 1994).

4.3. Microorganisms involved in nitrate-dependent Fe(II) oxidation identified with DNA-SIP

A strong effect of nitrate addition on acetate-assimilating microorganisms was found in the ^{13}C-acetate incubated soil (Figs. 3 and 4). In particular, the alternative electron acceptor changed the dominant functional microorganisms involved in soil redox processes in ^{13}C-heavy fractions (Fig. 5). For example, the nitrate- and Fe(III)-reducing bacteria were the dominant genera in the ^{13}C-acetate-only treatments, which was consistent with the content and sequential reduction of electron acceptors in soil. However, the addition of the NO_3^- provided sufficient electron acceptor for the growth of special microorganisms associated with NO_3^- reduction. The relative abundance of Fe(III)-reducing bacteria significantly decreased while that of NO_3^--reducing bacteria increased (Fig. 5). Compared with the treatments with NO_3^- only, the microbial community shifted toward the function of NO_3^- reduction coupled to Fe(II) oxidation in the treatments with NO_3^- and Fe(II). These dominant genera were nitrate-dependent Fe(II)-oxidizing bacteria (Fig. 5, Chen et al., 2018; Sperfeld et al., 2018). In the present study, many microorganisms were identified as putative responders to the NO_3^- or NO_3^- and Fe(II) addition based on the combination of high-throughput sequencing and SIP analysis.

In the treatments with NO_3^- only, the dominant bacteria affiliated with *Zoogloea* were previously described as NO_3^- reducers in a variety of anoxic environments, including sediments, activated sludge, and paddy soils (Unz, 2015; Li et al., 2016; Gao et al., 2018). It should be noted that although *Zoogloea* do not grow in the absence of NO_3^- under anoxic conditions, these bacteria can still survive under these conditions (Unz, 2015). Therefore, the relative abundance of *Zoogloea* could be maintained at a certain level without NO_3^- addition in the treatments with ^{13}C-acetate-only (Fig. 5). In addition, *Azospira*, *Pseudogulbenkiania*, and *Azoarcus* are also capable of NO_3^- reduction under anoxic conditions (Byrne-Bailey and Coates, 2012; Chen et al., 2018; Sperfeld et al., 2018). However, these microorganisms were significantly less abundant than *Zoogloea*, indicating that *Azospira*, *Pseudogulbenkiania*, and *Azoarcus* prefer acetate assimilation during NO_3^- reduction. *Geobacter* did not dominate despite ^{13}C-acetate being available for assimilation, possibly due to a thermodynamic inhibition of Fe(III) reduction caused by the addition of NO_3^- (Zhang et al., 2012).

In the treatments with NO_3^- and Fe(II), microbial NO_3^- reduction coupled with Fe(II) oxidation occurred (Fig. 1). However, the addition of Fe(II) decreased the rate of NO_3^- reduction, which was consistent with other nitrate-dependent Fe(II) oxidation processes (Klueglein and Kappler, 2013; Li et al., 2018). In the treatment with NO_3^-, the rate of NO_3^- reduction was 0.167 mM h^{-1} whereas the reduction rate decreased to 0.093 mM h^{-1} in the presence of Fe(II) (Fig. 1). It is possible that nitrate-dependent Fe(II)-oxidizing bacteria become encrusted with the minerals formed as Fe(II) oxidation proceeds, or that NO_3^- reductase is encrusted in the periplasm (Kappler et al., 2010; Klueglein et al., 2014). Previous reports have shown that different patterns of encrustation were observed during Fe(II) oxidation by nitrate-dependent Fe(II)-oxidizing bacteria, including a mineral-filled periplasm, a mineralized cytoplasm, and encrustation of the cell surface (Kappler et al., 2010; Klueglein et al., 2014). Under the influence of Fe(II), the most dominant genus in ^{13}C-heavy fractions was *Pseudogulbenkiania*, which has been widely found in paddy soil or rice–soybean rotation fields (Tago et al., 2011; Yoshida et al., 2012; Li et al., 2019). Many strains affiliated with *Pseudogulbenkiania* are capable of nitrate-dependent Fe(II) oxidation under anoxic conditions (Ishii et al., 2016). A previous study showed that *Pseudogulbenkiania* was the main contributor to the sequential reduction of NO_3^- to NO_2^-, followed by N_2O and ultimately production of N_2 (Li et al., 2019). Although N_2O and N_2 were not measured in the present study, the change of NO_2^- concentrations indicated that *Pseudogulbenkiania* was responsible for NO_3^- and NO_2^- reduction (Fig. 1). However, *Pseudogulbenkiania* was not enriched in the treatments with NO_3^- only in the ^{13}C-acetate-heavy fractions, suggesting that the addition of Fe(II) could stimulate the NO_3^- reducer, *Pseudogulbenkiania*. Tago et al. (2011) found that *Pseudogulbenkiania* strains were dominant in the paddy soils with reduced metals and N–fertilizer, indicating that *Pseudogulbenkiania* played a role in nitrate-dependent Fe(II)-oxidation. *Azoarcus* was the second most abundant genus in the ^{13}C-heavy fractions, and its relative abundance was significantly increased compared to the treatments with acetate only or NO_3^- (Fig. 5), suggesting that the addition of NO_3^- and Fe(II) could stimulate the growth of *Azoarcus*. Members affiliated with *Azoarcus* can assimilate a variety of organic matter as carbon sources under denitrifying conditions (Reinhold-Hurek et al., 2015). In addition, *Azoarcus* has been detected in reduced metal-rich lake sediments and paddy soils, such as soil rich in arsenic (III) and Fe(II) (Qiao et al., 2018; Pang et al., 2021). The genomic analysis of *Azoarcus* showed that it could encode several deca-heme cytochromes that were highly homologous with MtoA and MtoD c-type cytochromes associated with controlling the electron transfer during Fe(II) oxidation (Liu et al., 2018; Levett et al., 2020). These findings suggest that in the changes in reduced or oxidized substances could shift the microbial communities that assimilate ^{13}C-acetate under anoxic conditions.

In addition to the nitrate-dependent Fe(II) oxidizers discussed above, other notable genera for ^{13}C-acetate assimilation in the treatments with NO_3^- and Fe(II) were attributed to *Azospira*, *Zoogloea*, *Geothrix* and *Bdellovibrio* (Fig. 5 and S4). *Azospira* and *Zoogloea* were detected in the treatments with NO_3^- only as NO_3^- reducers under anoxic conditions. However, the relative abundances of these two genera significantly decreased with the addition of Fe(II), suggesting that Fe(II) might suppress the growth of these NO_3^- reducers. Members affiliated with the genus *Geothrix* are widely distributed in anoxic environments and act as Fe(III)-reducing bacteria (Nevin and Lovley, 2002; Xiong et al., 2022). In the present study, *Geothrix* may have reduced the dissolved Fe(III) formed by nitrate-dependent Fe(II) oxidizers using ^{13}C-acetate as an electron donor. A previous study reported that *Geothrix* could produce one or more electron shuttles that enhanced the electron transfer from bacteria to dissolved or solid Fe(III), which could promote Fe(III) reduction under denitrifying conditions (Zhu et al., 2018). Bacteria affiliated with *Bdellovibrio* have been isolated from a wide range of

environments, such as paddy soil, freshwater, and seawater (Ruby, 1992). Unfortunately, few ecological investigations have been conducted to determine their roles in various environments. A recent study showed that *Bdellovibrio* might be linked to some anaerobic respiration processes involving Fe(III) or SO_4^{2-} reduction (Bond et al., 2000). However, more studies are needed to investigate the functions of *Bdellovibrio* in various environments in the future.

5. Conclusions and implications

In the present study, DNA-SIP combined with 16S rRNA gene amplicon sequencing was applied to identify the diversity of the acetate-assimilating microbial community, especially the microorganisms closely associated with soil redox processes. The dominant genera, including *Geobacter*, *Azospira*, *Azospirillum*, *Ideonella*, and *Desulfovibrionia*, were capable of NO_3^-, Fe(III), and SO_4^{2-} reduction, which are the most important electron acceptors in flooded soils (Hori et al., 2010; Xu et al., 2019). The NO_3^- reduction and Fe(II) oxidation processes significantly affected the acetate-assimilating microbial community from the original soil. The enriched genera in ^{13}C-heavy fractions were associated with NO_3^- reduction and nitrate-dependent Fe(II) oxidation. In the treatments with NO_3^-, *Zoogloea*, *Azospira*, *Azoarcus*, and *Geothrix* were the dominant genera while *Pseudogulbenkiania*, *Azospira*, *Zoogloea*, *Azoarcus*, and *Bdellovibrio* dominated in the treatments with NO_3^- and Fe (II). Given the significant difference of enrichments in ^{13}C-heavy fractions in different treatments, *Zoogloea* and *Pseudogulbenkiani* might be key microorganisms associated with NO_3^- reduction and nitrate-dependent Fe(II) oxidation, respectively. In this study, the most dominant genera from the treatments with NO_3^- or NO_3^- and Fe(II) were analogous to those reported in previous studies of anoxic soil environments involved in N and Fe cycling, suggesting that heterotrophic NO_3^- reducers and nitrate-dependent Fe(II) oxidizers are widespread in soil environments (Li et al., 2016; Friedl et al., 2018; Liu et al., 2019). However, the use of ^{13}C-acetate as the only organic carbon might limit our understanding of the microorganisms involved in NO_3^- reduction and Fe(II) oxidation that prefer other carbon sources. Therefore, selecting a suitable carbon source to stimulate the growth of functional microorganisms could help to efficiently remove excess NO_3^- and Fe in soil. In addition, although DNA-SIP revealed which microorganisms played an important role in NO_3^- reduction coupled with Fe(II) oxidation, the understanding of NO_3^- reduction coupled with Fe(II) oxidation by these microorganisms is still limited. Further research, such as pure isolation or omics-related techniques, is needed and will improve the understanding of the metabolic processes and how these functional microorganisms influence the biogeochemistry of C, Fe, and N cycling in soils.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109059.

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