Soil Biology & Biochemistry 94 (2016) 70–79

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Changes in the composition and diversity of microbial communities during anaerobic nitrate reduction and Fe(II) oxidation at circumneutral pH in paddy soil

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ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form
19 November 2015
Accepted 20 November 2015
Available online 13 December 2015

Keywords:
Nitrate reduction
Fe(II) oxidation
Paddy soil
Circumneutral pH
Microbial community

ABSTRACT

Kinetics of nitrate (NO_3^-) reduction and ferrous iron (Fe(II)) oxidation in paddy soil were investigated under anoxic conditions at circumneutral pH using three different treatments (i.e., Lactate + Fe(II), Lactate + NO_3^-, and Lactate + NO_3^- + Fe(II)). The results revealed that NO_3^- could be rapidly reduced to nitrite (NO_2^-) within two days in treatments of Lactate + NO_3^- and Lactate + NO_3^- + Fe(II), and the presence of Fe(II) facilitated the NO_2^- reduction. Whereas no obvious Fe(II) oxidation was observed in treatment of Lactate + Fe(II), Fe(II) oxidation took place only when NO_3^- was added. Illumina high-throughput sequencing used to profile the diversity and abundance of microbial communities showed that the phyla of *Proteobacteria* and *Firmicutes* had a dominant presence in all three treatments with lactate. *Acidaminobacter*, *Proteiniclasticum*, *Alkaliphilus*, and *Natronincola_Anaerovirgula* were found to be the dominant genera during NO_3^- reduction without Fe(II) after addition of lactate, and all were seldom reported to be associated with NO_3^- reduction. *Azospira*, *Zoogloea*, and *Dechloromonas* dominated during NO_3^- reduction in the presence of Fe(II), and all are betaproteobacterial NO_3^--reducing bacteria that do not produce ammonium as end products of NO_3^- reduction. Whereas *Azospira*, *Zoogloea*, and *Dechloromonas* have been isolated or identified from NO_3^--reducing Fe(II) oxidation culture previously, the NO_2^- produced by these NO_3^- reducing bacteria can also oxidize Fe(II) abiotically, resulting in facilitated NO_2^- disappearance in the treatment of Lactate + NO_3^- + Fe(II). These findings increase our understanding of the processes of NO_3^- reduction in the absence and presence of Fe(II) in anoxic paddy soil at circumneutral pH and extend our knowledge of the microbial communities involved in these processes.

1. Introduction

Iron (Fe) is the fourth most abundant element in the Earth's crust as well as the most frequently utilized transition metal in the biosphere (Cornell and Schwertmann, 2003; Kappler and Straub, 2005). Redox transformations of Fe strongly influence the degradation and preservation of carbon and the fate of many nutrients and contaminants (Borch et al., 2010; Lalonde et al., 2012; Melton et al., 2014c). Ferrous iron (Fe(II)) is easily soluble and more bioavailable but is readily chemically oxidized by oxygen at circumneutral pHs in oxic environments. Fe(II) oxidation processes at circumneutral pH are mediated by neutrophilic Fe(II)-oxidizing bacteria, which include microaerophilic Fe(II)-oxidizing bacteria, anaerobic phototrophic Fe(II)-oxidizing bacteria and anaerobic nitrate (NO_3^-)-reducing Fe(II)-oxidizing bacteria, at the oxic–anoxic interface or in anoxic environments (Widdel et al., 1993; Straub et al., 1996; Emerson and Moyer, 1997).

Whereas the microaerophilic and phototrophic Fe(II) oxidizers are lithoautotrophic microorganisms, most of the NO_3^--reducing Fe(II) oxidizers isolated so far need an organic cosubstrate (e.g., acetate or succinate) for growth (Straub et al., 1996, 2004; Kappler et al., 2005). The NO_3^--reducing Fe(II) oxidizers also play an important role in the biogeochemical cycles of carbon (C) and nitrogen (N). For example, many NO_3^--reducing Fe(II) oxidizers can produce nitrous oxide (N_2O), and the accumulation of N_2O was considered to inhibit methanogenesis due to its toxic effect (Chidthaisong and Conrad, 2000; Straub et al., 2004; Kappler et al., 2005; Jones et al., 2015). NO_3^--reducing Fe(II) oxidation in the presence of fixed carbon has been documented for *Acidovorax*,

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http://dx.doi.org/10.1016/j.soilbio.2015.11.013
0038-0717/

Dechloromonas (Chakraborty and Picardal, 2013), *Desulfitobacterium* (Shelobolina et al., 2003), *Comamonas*, *Parvibaculum*, *Rhodanobacter* (Blöthe and Roden, 2009), *Ferroglobus* (Hafenbradl et al., 1996) and even *Escherichia coli* (Brons et al., 1991). These reported NO_3^--reducing Fe(II) oxidizers have been isolated from a wide range of habitats (even from a submarine hydrothermal system and hypersaline sediment) and are phylogenetically diverse, including α-, β- and γ-*Proteobacteria* and Archaea, indicating their environmental prevalence and importance (Kappler and Straub, 2005; Emmerich et al., 2012; Carlson et al., 2013).

Red soils, with a high Fe content of 3.61%, predominantly cover the tropical and subtropical regions of southern China and play an important role in rice production (Chen et al., 2014). Paddy soils are highly modified by anthropogenic activities with inputs and accumulation of organic C high as 31 g/kg and organic N as 2.3 g/kg (Chen et al., 2008; Kögel-Knabner et al., 2010; Ding et al., 2015). When switching from oxic conditions to anoxic conditions after flooding, anaerobic microorganisms can use alternative to oxygen electron acceptors (e.g., Fe(III) and NO_3^-) for the oxidation of organic matter (Wang et al., 2009; Kögel-Knabner et al., 2010). Fe(II) concentrations in excess of 10 mM are detected in flooded paddy soils of southern China (Li and Horikawa, 1997), and NO_3^- can be further reduced to nitrite (NO_2^-), nitric oxide (NO), N_2O and N_2 biologically via denitrification (Chidthaisong and Conrad, 2000; Kögel-Knabner et al., 2010; Thomson et al., 2012; Liu et al., 2014a). Therefore, it can be hypothesized that Fe(II) oxidation and NO_3^- reduction might have important contribution to the Fe/N cycles in paddy soils. Whereas most of the source materials for enrichment of NO_3^--reducing Fe(II) oxidizers are sediments from freshwaters, blackish waters, and marine sources (Straub et al., 1996; Straub and Buchholz-Cleven, 1998; Weber et al., 2006a,c; Melton et al., 2014a), few studies have investigated changes of microbial communities during NO_3^- reduction and Fe(II) oxidation in paddy soils.

In the current study, a paddy soil was collected as inoculum source from the tropical and subtropical regions of southern China. Our objectives were (i) to investigate the dynamics of NO_3^- reduction and Fe(II) oxidation in this paddy soil with additional organic substrate at circumneutral pH under anoxic conditions; and (ii) to profile the changes in diversity and abundance of microbial communities during the incubation using next generation sequencing. The results obtained are expected to extend our knowledge of NO_3^- reduction and Fe(II) oxidation in flooded paddy soil environments as well as distribution and diversity of bacteria involved in these processes.

2. Materials and methods

2.1. Soil sampling and chemicals

A soil sample was collected from 15 cm beneath the soil surface of the paddy soil in the Cuiheng Village of Zhongshan City, China (22° 28′ 12.58″ N, 113° 32′ 33.51″ E), in August 2011. Once taken, the sample was extruded into glass tubes, stoppered, and immediately transported back to the laboratory and stored in an anaerobic chamber to be maintained under anoxic conditions for one day before the experiments. The soil contained (weight/weight) 54% SiO_2, 21% Al_2O_3, 23 g/kg total Fe, 35 g/kg total organic carbon and 44% moisture content, and was identified as Inceptisol according to the United States Department of Agriculture (USDA) soil taxonomy (Soil Survey Staff, 2014). Containing (weight/weight) clay (35%), sand (40%) and silt (25%), the soil was classified as clay loam, based on the USDA soil textural triangle standard. Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES, ≥99%) and sodium lactate (≥99%) were purchased from Sigma–Aldrich (USA). $NaNO_3$ (≥99%), $FeSO_4 \cdot 7H_2O$ (≥99%), and other analytical-grade chemicals were purchased from the Guangzhou Chemical Co.

2.2. Batch experimental procedures

Strict anaerobic techniques and sterile conditions (Li et al., 2009) were used throughout the NO_3^--reducing Fe(II) oxidation experiment. Three batch treatments were conducted with details presented in Table 1. Since each sampling needed to sacrifice triplicate serum bottles in each treatment, total 100 replicates were conducted for each treatment. A homogenized paddy soil slurry was prepared by mixing the soil with sterile deionized water, 1 mL portions of which were added to sterile serum bottles containing 30 mL of 20 mM PIPES buffer (pH = 7.0) to achieve a final soil (dry weight):water ratio of 1:100. Lactate was added as a carbon source with an initial concentration of 10 mM. The initial concentrations of NO_3^- and Fe(II) were 15 mM and 5 mM, respectively. In order to exclude the interference of O_2 as competitive electron acceptor, the cultures in the serum bottles were purged with O_2-free N_2 gas for 30 min and sealed with Teflon-coated butyl rubber stoppers and crimp seals, and all vials were incubated in a Bactron Anaerobic/Environmental Chamber II (Shellab, Sheldon Manufacturing Inc., Cornelius, OR) at 30 °C in the dark.

2.3. Chemical analytical methods

During the incubation period, triplicate bottles were sacrificed for chemical analysis at regular intervals with each chemical determination of each bottle performed without technical replicate. To determine NO_3^-, NO_2^- and NH_4^+, an aliquot of sample was first centrifuged at 8500 × g for 20 min to remove soils and exposed to O_2 to rapidly oxidize Fe(II), followed by filtering using a syringe filter containing a 0.22-μm mixed cellulose ester membrane as previously described (Liu et al., 2014a). The concentrations of NO_3^- and NO_2^- were determined in the filtrate by ion chromatography (Dionex ICS-90) with an ion column (IonPac AS14A 4 × 250 mm). A mobile phase containing Na_2CO_3 (8 mM) and $NaHCO_3$ (1 mM) was operated at a flow rate of 1 mL min^{-1}. The detection limits by the ion chromatography analysis were approximately 0.003 mM for both NO_3^- and NO_2^-. The concentration of NH_4^+ was measured using spectrophotometry at 420 nm after a colorimetric reaction with Nessler's reagent (Paul et al., 2007). Before Nessler's reagent was added, an aliquot of the sample to be analyzed was diluted with ultrapure water. Ammonium chloride solution was used as a standard. The concentrations of dissolved and 0.5 M HCl-extractable Fe(II) were determined using 1,10-phenanthroline colorimetric assay as previously described (Li et al., 2010).

2.4. DNA extraction, 16S rRNA gene amplification, and high throughput sequencing

The soil was collected from the serum bottle by centrifugation at 10,000 × g for 10 min. The genomic DNA was extracted from the soil sample using a PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's instructions. The V3–V4 hypervariable region of bacterial and archaeal 16S rRNA

Table 1
Experimental setups of the three treatments with lactate. Total 100 replicates were conducted for each treatment.

Treatment	Soil	Lactate	Fe(II)	NO_3^-
(a) Lactate + Fe(II)	0.3 g	10 mM	5 mM	–
(b) Lactate + NO_3^-	0.3 g	10 mM	–	15 mM
(c) Lactate + NO_3^- + Fe(II)	0.3 g	10 mM	5 mM	15 mM

genes was amplified using the universal primer set of F338 (5′-ACTCCTACGGGAGGCAGCA-3′) and R806 (5′-GGACTACVSGGGT ATCTAAT-3′), with a sample-specific 12-bp barcode added to the reverse primer. This pair of primers was selected as it covers a broad taxonomic range with high target coverage (>90%) for majority of the prokaryotic phyla (Lee et al., 2012). Each DNA sample was amplified in triplicate in 30 μL reaction mixtures using the following PCR program: initial denaturation at 95 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 1 min, extension at 72 °C for 1 min; and a final extension of 10 min at 72 °C. Replicate amplicons were pooled for purification with an E.Z.N.A.® Gel Extraction Kit (OMEGA Biotek, USA). A single composite sample was prepared by combining approximately equimolar amounts of purified PCR products from each sample and then sequenced on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) with paired-end (PE) 250 bp sequencing kits.

2.5. Analysis of microbial community composition and diversity

Raw Illumina PE 250 bp data were first quality controlled using in-house perl scripts (See Supporting Information), to remove reads with ambiguous bases and were trimmed at the 3′ end to eliminate continued bases with a quality score <20. Then, the paired-end reads were combined using the Flash software with default parameters (Magoč and Salzberg, 2011) to obtain the full V3–V4 hypervariable region of bacterial and archaeal 16S rRNA genes sequences. The obtained sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software pipeline (Caporaso et al., 2010b). The chimeric and low quality sequences were identified and removed, whereas the 12-bp barcode was examined in order to assign sequences to individual samples. Operational taxonomic units (OTUs) were identified with uclust at the 97% sequence similarity level (Edgar, 2010), and a representative sequence from each OTU was aligned using PyNAST (DeSantis et al., 2006; Caporaso et al., 2010a). Taxonomic classification of each OTU was determined using the Ribosomal Database Project (RDP) classifier (Wang et al., 2007). Relative abundance (%) of individual taxa within each community was estimated by comparing the number of sequences assigned to a specific taxon versus the number of total sequences obtained for that sample. The alpha microbial biodiversity of the samples was estimated by the abundance-based indices of Chao1, Shannon and Simpson. Beta diversity (unweighted and weighted UniFrac distances) was calculated on the basis of a subset of 29,070 randomly selected sequences per community. The genomic datasets have been deposited in the European Nucleotide Archive under accession number PRJEB11487.

3. Results and discussion

3.1. Kinetics of NO_3^- transformation

In order to investigate the individual effect of exogenous Fe(II) and NO_3^- on anaerobic NO_3^- reduction and Fe(II) oxidation at circumneutral pH in paddy soil, two treatments of Lactate + Fe(II) and Lactate + NO_3^- were performed. In the treatment of Lactate + Fe(II), 5 mM of Fe(II) was added initially without any addition of NO_3^-. The results in Fig. 1a revealed that whereas the concentrations of NO_3^- and NO_2^- did not change over time and were maintained at a low level of 0.22 mM and 0.01 mM, respectively, the concentration of NH_4^+ was detected as 1.21 mM at the beginning as background, and then decreased over time. Ammonia can be oxidized to NO_2^- coupled to dissimilatory Fe(III) reduction or oxidized to N_2 gas by anaerobic ammonia-oxidizing bacteria using NO_2^- as an electron acceptor under anoxic conditions (Clément et al., 2005; Zhu et al.,

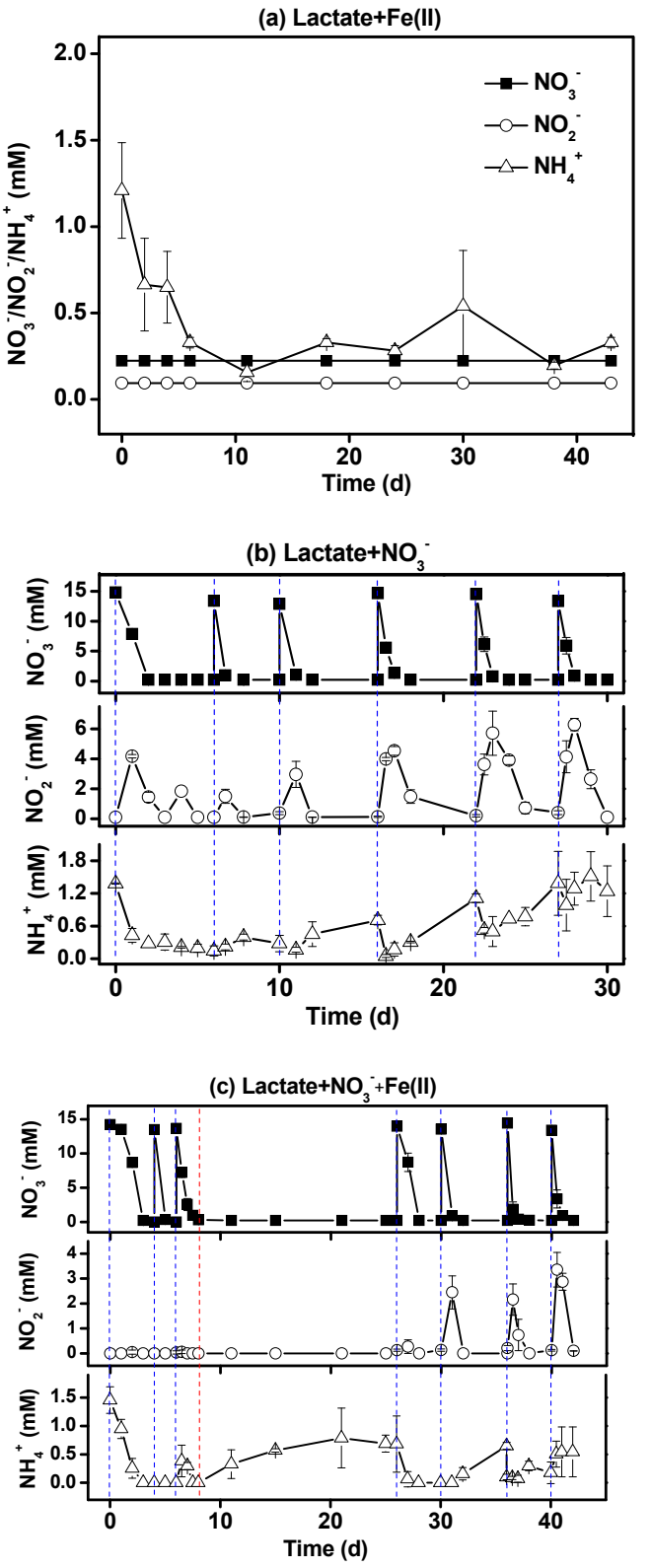

Fig. 1. Changes in NO_3^-, NO_2^- and NH_4^+ concentrations with time in different treatments. (a) Lactate + Fe(II); (b) Lactate + NO_3^-; (c) Lactate + NO_3^- + Fe(II). The blue vertical dash lines indicate the times for the addition of NO_3^- and lactate, and the red vertical dash lines indicate the times for the addition of lactate only. The concentrations of lactate, Fe(II) and NO_3^- added each time were 10 mM, 5 mM and 15 mM, respectively. Error bars represent standard deviation from the mean (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2011). However, no change was observed in NO_2^- concentration (Fig. 1a), hence the ammonium might only serve as a nitrogen source for soil bacterial assimilation metabolism (Shrestha, 2008).

As shown in Fig. 1b, a total of six cycles were carried out where fresh NO_3^- (15 mM) and lactate (10 mM) was added each time in the treatment of Lactate + NO_3^-. Once NO_3^- was added into the reaction soil suspension, NO_3^- was reduced completely within 2 days. Along with the decrease of NO_3^-, the NO_2^- concentrations increased and then decreased after 2 days. The NH_4^+ concentrations were approximately 0.6 mM during the first three cycles but increased gradually to approximately 1.5 mM at the end of the incubation. Whereas many NO_3^--reducing bacteria are able to reduce NO_3^- to ammonium under anoxic conditions (Zhang et al., 2012; Liu et al., 2014a) and dissimilatory NO_3^- reduction to ammonium bacteria have also been found in different paddy soils (Rütting et al., 2011), only <8% of the NO_3^- was reduced to ammonium in the Lactate + NO_3^- treatment group. NO_2^- as the first intermediate during microbial NO_3^- reduction process can be further reduced to NO, N_2O and N_2 via denitrification (Kögel-Knabner et al., 2010; Thomson et al., 2012; Liu et al., 2014b; Zhang et al., 2014). Therefore, the denitrification processes were supposed to happen in the Lactate + NO_3^- treatment group though we haven't quantified the further denitrification products. In addition, results of the treatment of NO_3^- without lactate showed that no NO_3^- was reduced (Fig. S1d), suggesting that lactate as carbon source is necessary for the microbial NO_3^- reduction. No lactate was detected at the end of each cycle in the Lactate + NO_3^- treatment group (data not shown), which indicated that lactate was completely consumed by the NO_3^--reducing bacteria. It has been reported that lactate ($C_3H_5O_3^-$) can be oxidized to CO_2 and HCO_3^- via intermediates (e.g., pyruvate and acetate) by microbes (Pankhania et al., 1988), and NO_3^- can be reduced to N_2 and/or NH_4^+ by microorganisms under anoxic conditions (Melton et al., 2014b). Herein, microbial NO_3^- reductions to NH_4^+ and denitrification reactions coupled to lactate oxidation were described as Eqs. (1) and (2), respectively.

$$8C_3H_5O_3^- + 12NO_3^- + 24H^+ \rightarrow 16CO_2 + 8HCO_3^- + 12NH_4^+ + 4H_2O \quad (1)$$

$$5C_3H_5O_3^- + 12NO_3^- + 12H^+ \rightarrow 10CO_2 + 5HCO_3^- + 6N_2 + 16H_2O \quad (2)$$

In the treatment group of Lactate + NO_3^- + Fe(II), Fe(II) solution was only added at the beginning, and a total of seven cycles were carried out with fresh NO_3^- (15 mM) and lactate (10 mM) added each time (Fig. 1c). During 0–8 d, complete NO_3^- reduction was observed quickly after NO_3^- was added; however, no NO_2^- production was detected simultaneously, suggesting that the presence of Fe(II) facilitated NO_2^- reduction, which is consistent with that from Weber et al. (2006b). In most previously reported NO_3^--reducing Fe(II)-oxidizing cultures, NO_2^- was further reduced to NO, N_2O and N_2 via denitrification as shown in Eq. (3), or via chemo-denitrification as shown in Eq. (4) in which NO_2^- can oxidize Fe(II) abiotically at circumneutral pH particularly in the presence of minerals, cell surfaces or chelators (Ratering and Schnell, 2001; Klueglein and Kappler, 2013; Klueglein et al., 2014a; Melton et al., 2014c; Jones et al., 2015). A gradual decrease of NH_4^+ was observed in the Lactate + NO_3^- + Fe(II) treatment group, similar to the results of the other two treatments with lactate (Fig. 1a and b), which is probably attributed to the assimilation process as well.

$$10Fe(II) + 2NO_3^- + 24H_2O \rightarrow 10Fe(OH)_3 + N_2 + 18H^+ \quad (3)$$

$$4Fe(II) + 2NO_2^- + 5H_2O \rightarrow 4FeOOH + N_2O + 6H^+ \quad (4)$$

As most of the added Fe(II) had been oxidized during 0–8 d (Fig. 2c), only lactate (10 mM), not NO_3^-, was added on the 8th day in order to stimulate anaerobic Fe(III) reduction, resulting in a recovery of Fe(II). During 8–26 d, the concentration of NH_4^+ increased gradually to approximately 0.8 mM, which might be attributed to nitrogen mineralization as NH_4^+ is the dominant nitrogen species during nitrogen mineralization in paddy soils under anaerobic conditions (Li et al., 2003). During 26–42 d, another four cycles with additions of fresh NO_3^- (15 mM) and lactate (10 mM) each time were performed after most of the Fe(III) was reduced to Fe(II) (Fig. 2c). NO_3^- was reduced within two days with an average reduction rate ± standard deviation (SD) of 2.0 ± 0.045 d^{-1}, and NO_2^- was re-formed during the last three cycles. Moreover, in addition to the ammonia assimilation, the anaerobic ammonium oxidation by Fe(III) may also account for the disappearance of NH_4^+ in this system (Mulder et al., 1995; Clément et al., 2005).

3.2. Kinetics of Fe(II) transformation

With the addition of 5 mM Fe(II) in the Lactate + Fe(II) treatment, the concentrations of HCl-extractable Fe(II) did not change evidently over time (Fig. 2a) despite the slight fluctuation for dissolved Fe(II), suggesting that no anaerobic Fe(II) oxidation took place in the absence of NO_3^-. The results in Fig. 2b revealed that the background concentrations of dissolved Fe(II) and HCl-extractable Fe(II) were 0.05 mM and 0.7 mM in the soil suspension of Lactate + NO_3^- treatment group, respectively. The dissolved Fe(II) did not change significantly, and the HCl-extractable Fe(II) only slightly decreased over time during the incubation period. As a result, the observed NO_3^- reduction in Fig. 1b is considered to be mainly driven by NO_3^--reducing bacteria. Even if microbial NO_3^--dependent Fe(II) oxidation happened in this treatment, the 0.7 mM of HCl-extractable Fe(II) could only reduce maximum 0.14 mM of NO_3^- according to the stoichiometry of microbial NO_3^--dependent Fe(II) oxidation reaction (Eq. (3)).

In the treatment group of Lactate + NO_3^- + Fe(II), the concentrations of both dissolved Fe(II) and HCl-extractable Fe(II) decreased substantially during the first three cycles (Fig. 2c), indicating that the presence of NO_3^- induced Fe(II) oxidation via biotic (catalyzed by bacteria) or abiotic reaction (chemo-denitrification) as compared with that no Fe(II) oxidation was observed in the Lactate + Fe(II) treatment (Fig. 2a). Since lactate was added as organic substrate and dissolved Fe(II) was present as the predominant form during this period, it is believed that the observed NO_3^- reduction was mainly coupled to oxidation of lactate and dissolved Fe(II). During the Fe(II) recovery phase (8–26 d), the concentrations of HCl-extractable Fe(II) increased gradually and returned to the same level as observed at the beginning of the experiment, but the concentrations of dissolved Fe(II) only slightly increased from 0 mM to approximately 2 mM. These results suggest that most of the Fe(II) species were present as cell-associated Fe(II) or mineral-associated Fe(II) after Fe(III) reduction. During the last four cycles, whereas the concentrations of dissolved Fe(II) did not change markedly, the concentrations of HCl-extractable Fe(II) only decreased slightly from 5.5 mM to around 4.0 mM where sorbed Fe(II) or mineral-associated Fe(II) accounted for approximately 70% of the HCl-extractable Fe(II), which is analogous to previous studies (Weber et al., 2006c; Coby et al., 2011). These results suggest that the observed NO_3^- reduction (Fig. 1c) during the last four cycles may be mainly induced through the NO_3^- reduction by microorganisms and possibly coupled to the oxidation of cell-associated Fe(II) or mineral-associated Fe(II) as well. Coby et al. (2011) reported that when inputs of organic C are high compared to NO_3^-, organic C oxidation by NO_3^- reducing bacteria exhausts available NO_3^-, thus

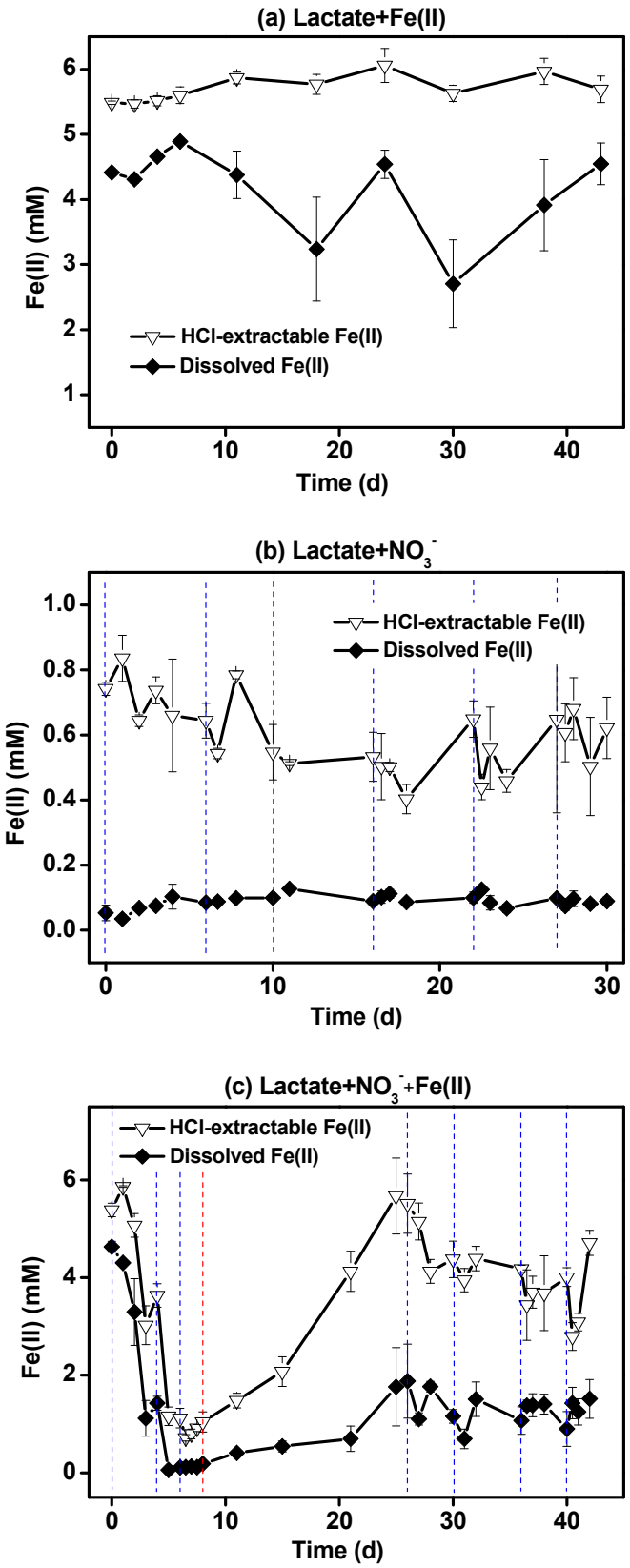

Fig. 2. Changes in 0.5 M HCl-extractable Fe(II) and dissolved Fe(II) concentrations with time in different treatments. (a) Lactate + Fe(II); (b) Lactate + NO_3^-; (c) Lactate + NO_3^- + Fe(II). The blue vertical dash lines indicate the times for the addition of NO_3^- and lactate, and the red vertical dash lines indicate the times for the addition of lactate only. The concentrations of lactate, Fe(II) and NO_3^- added each time were 10 mM, 5 mM and 15 mM, respectively. Error bars represent standard deviation from the mean (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

allowing microbial Fe(III) reduction to become the predominant terminal electron-accepting pathway. If NO_3^- was completely oxidized to NH_4^+ or N_2 during the microbial NO_3^- reduction, 10 mM or 6.25 mM of lactate is required according to Eqs. (1) and (2). Since only <5% of NO_3^- were converted to NH_4^+ in the last four cycles of the Lactate + NO_3^- + Fe(II) treatment, the excess addition of lactate can explain why there was a slight increase of Fe(II) once NO_3^- was completely reduced in each cycle with the addition of lactate and NO_3^-.

In addition, results in Fig. S1e revealed that no evident NO_3^- reduction or Fe(II) oxidation was observed in the treatment of NO_3^- + Fe(II) without lactate, indicating that Fe(II) oxidation did not happen in the absence of lactate and NO_3^-. No lactate was detected at the end of each cycle in the Lactate + NO_3^- + Fe(II) treatment group (data not shown), suggesting that lactate was completely removed during the NO_3^- reduction and Fe(II) oxidation processes. Previous studies also reported that most of the NO_3^--reducing Fe(II) oxidizers isolated so far need an organic cosubstrate for growth (Straub et al., 1996, 2004; Kappler et al., 2005). Some NO_3^--reducing Fe(II) oxidizers can also use CO_2 as the sole carbon source (Weber et al., 2006b), which would not happen in this study since no CO_2 was added in the reaction system. The addition of lactate in the treatment of Lactate + NO_3^- + Fe(II) may not only stimulate the NO_3^--reducing bacteria but also the NO_3^--dependent Fe(II) oxidizers. As a result, both the microbial NO_3^--dependent Fe(II) oxidation process (Eq. (3)) and the abiotic chemodenitrification process (Eq. (4)) after microbial NO_3^- reduction might contribute to the overall NO_3^- reduction and Fe(II) oxidation.

At the beginning and the end of the incubation of each treatment, pH values were also measured with results presented in Fig. S2. While the pH values did not changed markedly in the treatments of Lactate + Fe(II), NO_3^- and NO_3^- + Fe(II), the pH values of the treatments of Lactate + NO_3^- and Lactate + NO_3^- + Fe(II) sustainably increased to approximately pH 9 at the end of incubation, suggesting that such an increase may be associated with the addition of lactate and NO_3^-. Similar increase of pH has been also observed in the NO_3^- reduction layer of natural sediments and flooded paddy soils (Kögel-Knabner et al., 2010; Melton et al., 2014b). Both of the reactions between lactate and NO_3^- (Eqs. (1) and (2)) can result in consumption of H^+, leading to an increase of pH of the reaction system. For 15 mM of NO_3^- added, it has the potential to consume 30 mM (Eq. (1)) and 15 mM (Eq. (2)) of H^+, respectively. Therefore, the pH increase in the Lactate + NO_3^- treatment may be attributed to the formation of N_2 or NH_4^+ accompanied by the consumption of H^+.

In the treatment of Lactate + NO_3^- + Fe(II), microbial mediated NO_3^--dependent Fe(II) oxidation presented as Eq. (3) can produce H^+ if this reaction did happen. However, even all the Fe(II) added (5 mM) was consumed via Eq. (3), the H^+ produced as 9 mM was still lower than the H^+ consumed as 30 mM or 15 mM via Eq. (1) or (2). On the other hand, if all the Fe(II) only reacted with NO_2^- abiotically via Eq. (4) after microbial NO_3^- reduction, there was only 7.5 mM of H^+ produced. It should be noted that Fe(II) was added only at the beginning with 5 mM, but NO_3^- was added seven times with total concentration of 105 mM during the incubation. Even microbial NO_3^--dependent Fe(II) oxidation and/or chemodenitrification did happen in this treatment particularly at the first two cycles (Figs. 1c and 2c), the repeated addition and reduction of NO_3^- definitely can lead to an sustainable increase of pH at the end of incubation.

3.3. Microbial community diversity and composition

As NO_3^- or/and Fe(II) with lactate was added in the soil suspension during the reaction period, the microbial community could have been acclimated and changed over time. The Illumina high-throughput sequencing generated a total of 578,791 quality sequences for the 15 analyzed samples, with the sequences for individual samples ranging from 29,070 to 45,307 (Table S1). In total, 8623 OTUs were identified in the complete data set, with an average of 1731 OTUs per sample. Over 99% of the OTUs could be assigned to a taxonomic group (phylum), and over 80% could be identified at the order level.

The dominant phyla in the raw soil samples without any amendment (0 d) were *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Acidobacteria*, *Planctomycetes* and *Actinobacteria* (relative abundance >3% for each phylum), whose sequences accounted for >85% of the total (Fig. 3). It is clear that the phyla of *Proteobacteria* and *Firmicutes* dominated in the three treatments with lactate after amendment, which is consistent with previous studies investigating iron oxidation and NO_3^- reduction (Weber et al., 2006a; Coby et al., 2011; Melton et al., 2014c). However, the relative abundance of the different phyla varied considerably. In the Lactate + Fe(II) treatment, the relative abundances of *Proteobacteria* and *Firmicutes* increased from 34% to 58%, and from 12% to 18%, respectively, at the end of incubation, whereas those of the rest of the phyla evidently decreased (Fig. 3a). In the Lactate + NO_3^- treatment group, the relative abundances of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* increased significantly after the addition of NO_3^-, whose sequences accounted for >95% of the total during the whole incubation period (Fig. 3b). In the treatment group of Lactate + NO_3^- + Fe(II), the phylum of *Proteobacteria* with relative abundances of 86–88% dominated during the first two cycles (0–6 d) (Fig. 3c). However, the relative abundances of *Firmicutes* and *Bacteroidetes* increased markedly from 11% to 27%, and from 0.86% to 3.2%, respectively, at the end of the Fe(II) recovery phase (26 d). During the last four cycles with the addition of NO_3^- and lactate per cycle, the phyla of *Proteobacteria*, *Firmicutes* and *Bacteroidetes* accounted for an average relative abundance of 96%, which is similar to that in the Lactate + NO_3^- treatment group.

At the genus level, the dominant genera in the raw soil samples before any amendment were *Azospira*, *Desulfobulbus*, *Desulfovibrio*, *Anaeromyxobacter*, *Rhodoplanes*, *Acidaminobacter*, *Proteiniclasticum* and *Alkaliphilus* (relative abundance >1%) (Table S2). The microbial composition also differed greatly among the three treatments with lactate and displayed a distinct succession (Table S2). In the Lactate + Fe(II) treatment group, *Desulfobulbus* dominated at the end of the incubation with a high relative abundance of 39%, and the relative abundance of *Desulfovibrio* evidently increased as well (Fig. 4a). Both *Desulfobulbus* and *Desulfovibrio* are sulfate-reducing anaerobes that can use lactate as an electron donor for sulfate reduction at a neutral pH (Kuever et al., 2014a,b). In this study, as ferrous sulfate was added as the source of Fe(II), it is reasonable to detect such a high relative abundance of these two sulfate reducers. In addition, some *Desulfovibrio* species have been reported to be capable of iron reduction as well (Liu et al., 2012; Kuever et al., 2014b), which may be responsible for the iron reduction in the Lactate + Fe(II) treatment group.

In the Lactate + NO_3^- treatment group (Fig. 4b), the relative abundances of *Azospira*, *Zoogloea*, *Dechloromonas*, *Acidaminobacter*, and *Proteiniclasticum* evidently increased after the 1st cycle (0–6 d). The relative abundances of *Azospira* and *Zoogloea* retained an average value ± SD of 4.3 ± 0.63% and 1.6 ± 0.19%, respectively, during the 2nd–5th cycle (10–27 d). The relative abundance of *Dechloromonas* decreased dramatically from 23% to 4.6% after the 2nd cycle (10 d) and maintained an average value ± SD of 2.8 ± 0.22% during the 3rd-5th cycle (16–27 d). Whereas the relative abundance of *Acidaminobacter* decreased to an average value ± SD of 7.3 ± 3.4% during the 3rd–5th cycle (16–27 d), those of *Proteiniclasticum*, *Alkaliphilus*, and *Natronincola_Anaerovirgula*

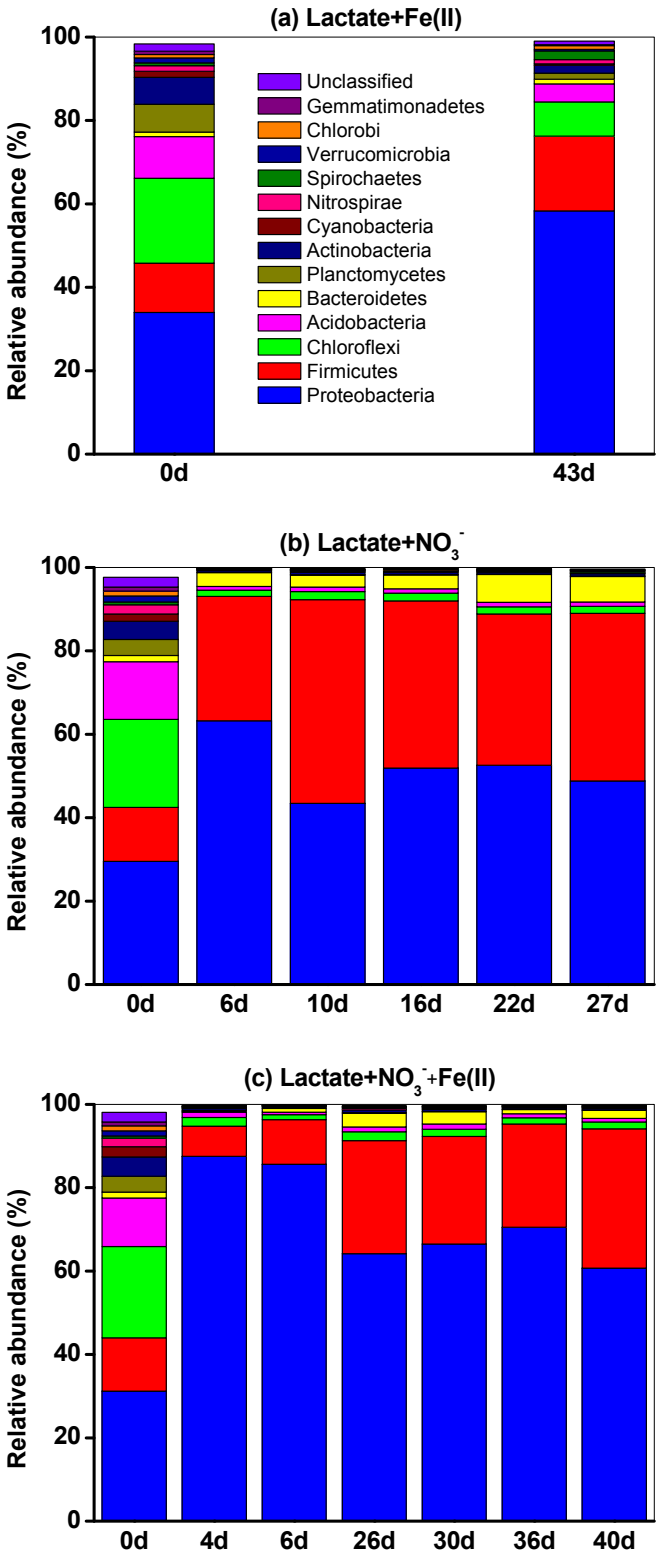

Fig. 3. Relative abundances (%) of the dominant microbial phyla with time dependence in different treatments revealed by 16S rRNA Illumina high-throughput sequencing. (a) Lactate + Fe(II); (b) Lactate + NO_3^-; (c) Lactate + NO_3^- + Fe(II). Those phyla with an average relative abundance of >0.5% in at least one sample were defined as the dominant phyla.

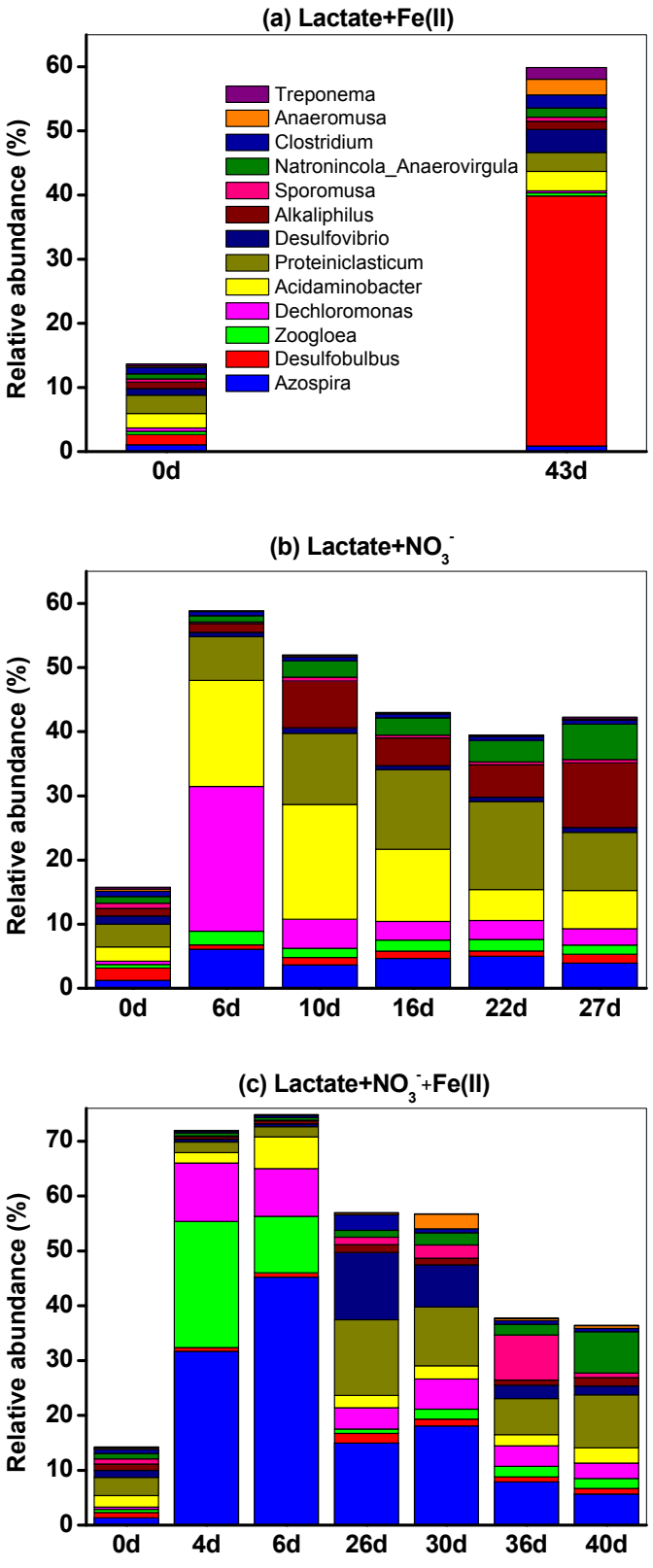

Fig. 4. Relative abundance (%) of the dominant microbial genera with time dependence in different treatments. (a) Lactate + Fe(II); (b) Lactate + NO_3^-; (c) Lactate + NO_3^- + Fe(II). Those genera with an average abundance of >1% in at least one sample were defined as the dominant genera.

increased to an average value ± SD of 12 ± 2.0%, 6.7 ± 2.6%, and 3.5 ± 1.4%, respectively, during the 2nd–5th cycle (10–27 d). It is obvious that *Acidaminobacter*, *Proteiniclasticum*, *Alkaliphilus*, and *Natronincola_Anaerovirgula* were the dominant genera during the NO_3^- reduction in the Lactate + NO_3^- treatment group, most of which belong to *Clostridiaceae* family.

Dechloromonas has been reported as a NO_3^--reducing bacterium with optimal growth pH at 7.0–7.2 (Horn et al., 2005; Wolterink et al., 2005). *Acidaminobacter*, *Proteiniclasticum*, *Alkaliphilus* and *Natronincola* can grow at a pH range of 6.7–8.8, 5.6–8.7, 5.5–12.5 and 7.5–10.2, respectively, but none of them has been reported to be able to use NO_3^- as terminal electron acceptor (Stams and Hansen, 1984; Zhilina et al., 2009; Zhang et al., 2010; Ben Aissa et al., 2015). The decreases of relative abundance of *Dechloromonas* and *Acidaminobacter* as well as the increase of relative abundance of *Proteiniclasticum*, *Alkaliphilus* and *Natronincola* may be associated with the increase of pH of the reaction system.

In the treatment group of Lactate + NO_3^- + Fe(II) (Fig. 4c), the relative abundances of *Azospira*, *Zoogloea*, and *Dechloromonas* increased sharply to an average value ± SD of 39 ± 9.6%, 17 ± 9.0%, and 9.7 ± 1.4%, respectively, during the 1st and 2nd cycles (0–6 d) for NO_3^- reduction and Fe(II) oxidation. All of these genera are associated with the *Rhodocyclaceae* family under the *Betaproteobacteria* class. All known betaproteobacterial NO_3^--reducing Fe(II) oxidizers produce N_2 rather than ammonium as the end product of NO_3^- reduction (Weber et al., 2006a; Coby et al., 2011), which is consistent with the results obtained here that no ammonium was detected in our system. *Azospira* and *Zoogloea* have been previously reported to have NO_3^- reduction and nitrogen-fixing capabilities (Bae et al., 2007; Shao et al., 2009; Oosterkamp et al., 2011). It should be noted that *Azospira* has been reported as the first anaerobic NO_3^--dependent Fe(II) oxidizer outside the *Diaphorobacter* and *Acidovorax* genera for which there is a completed genome sequence (Byrne-Bailey and Coates, 2012). In addition, the heterotrophic denitrifier *Dechloromonas* was also demonstrated to be capable of NO_3^--reducing Fe(II) oxidation in Fe redox cycling cultures at circumneutral pH (Weber et al., 2006a; Coby et al., 2011; Chakraborty and Picardal, 2013). The fast NO_3^- reduction and Fe(II) oxidation observed during the first three cycles, suggests that *Azospira*, *Zoogloea* and *Dechloromonas* play important roles in the NO_3^- reduction and Fe(II) oxidation in the soil suspension with NO_3^- and Fe(II).

At the end of the Fe(II) recovery phase (26 d), *Desulfovibrio* and *Proteiniclasticum* became the dominant genera with a relative abundance of 12% and 14%, respectively, whereas *Azospira* still retained a relatively high abundance of 15%. *Desulfovibrio*, a well-known sulfate-reducing bacterium, is also capable of coupling organics or H_2 oxidation to Fe(III) reduction, particularly when lactate is the organic substrate (Lentini et al., 2012; Liu et al., 2012; Roden et al., 2012). During 26–40 d for the NO_3^- reduction, the relative abundances of *Azospira* and *Desulfovibrio* on day 40 decreased to 5.7% and 1.6%, respectively, whereas that of *Natronincola_Anaerovirgula* increased to 7.6% and that of *Proteiniclasticum* maintained an average value ± SD of 9.0 ± 2.2%. Only a few studies have reported that the members of *Clostridiaceae* family are associated with NO_3^- reduction or iron transformation (Stackebrandt et al., 2014). As *Proteiniclasticum* was found in the raw soil sample and had a relatively high abundance during NO_3^- reduction in the treatment groups of Lactate + NO_3^- and Lactate + NO_3^- + Fe(II), *Proteiniclasticum* potentially acclimated to being involved in NO_3^- reduction in the paddy soil suspension with NO_3^-.

Based on the aforementioned NO_3^- and Fe(II) transformation kinetics and the microbial communities involved, an overall mechanism can be proposed as shown in Fig. 5. In a paddy soil system with lactate, Fe(II) and NO_3^-, *Azospira*, *Zoogloea* and

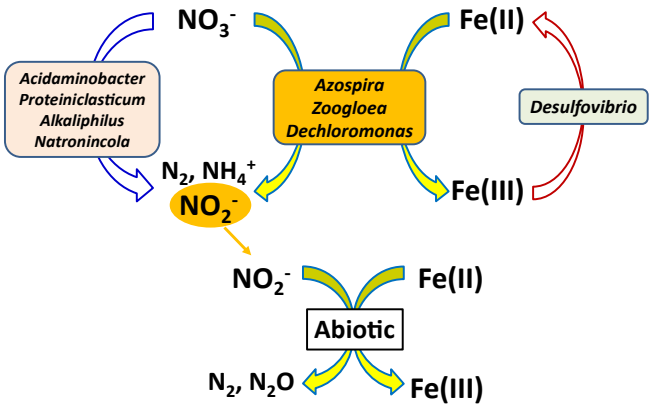

Fig. 5. A summary of the microbial communities involved in the processes of NO_3^- reduction, NO_3^- reduction and Fe(II) oxidation, and Fe(III) reduction in the tested paddy soil. N_2 was not quantified in this study.

Dechloromonas play dominant roles in the NO_3^- reduction coupled to lactate and Fe(II) oxidation, within which the abiotic chemodenitrification should be involved in; during Fe(II) deficiency, *Acidaminobacter*, *Proteiniclasticum*, *Alkaliphilus*, and *Natronincola_Anaerovirgula* were the dominant genera for NO_3^- reduction; and during NO_3^- deficiency, iron reduction by *Desulfovibrio* is a key process. In the natural paddy soil system, the input/output of alternative electron acceptors (e.g., Fe and NO_3^-) is accompanied with changes in functional microbial communities, and hence, the dynamics of NO_3^-/Fe and microbial communities with addition of lactate in this study will provide a clear picture describing the biogeochemical processes of iron and nitrogen transformation.

3.4. Environmental implications

Our experiment with the Lactate + NO_3^- + Fe(II) treatment shows that a microbial system undergoing repeated temporal oscillations in the input of organic substrate and NO_3^- developed a community structure capable of mediating NO_3^- reduction and Fe(II) oxidation, Fe(III) reduction, and NO_3^- reduction at circumneutral pH in paddy soil. Most of the dominant microorganisms identified to be responsible for NO_3^- reduction and Fe(II) oxidation in this study (i.e., *Zoogloea* and *Dechloromonas*) are analogous to those of previous experiments of anaerobic redox cycling of iron with freshwater sediment (Weber et al., 2006c; Coby et al., 2011), indicating that these NO_3^--reducing Fe(II) oxidizers are widespread in natural environments. The NO_3^--reducing Fe(II) oxidizers enriched in the tested paddy soil seem to prefer dissolved Fe(II) rather than sorbed Fe(II) during the first three cycles of NO_3^- reduction and Fe(II) oxidation process, which may be due to the high inputs of organic substrate in which organotrophic NO_3^- reduction dominates. Mineral-associated Fe(II) may be preferred by lithotrophic NO_3^--reducing Fe(II) oxidizers when organic substrate loading is limited (Coby et al., 2011). Most of the dominant genera in the raw paddy soil have been enriched during the three different treatments, which include *Azospira*, *Desulfobulbus*, *Desulfovibrio*, *Acidaminobacter*, *Proteiniclasticum* and *Alkaliphilus*. This suggests that functional microorganisms for NO_3^- reduction and Fe(II) oxidation from the natural paddy soils can be readily developed in the presence of Fe(II) and NO_3^-, once the soils are switched from oxic to anoxic conditions. In addition, the microbial communities found in the paddy soil for Fe(III) reduction and NO_3^- reduction are different from those found in previous studies with sediments as inocula (Weber et al., 2006a; Coby et al., 2011; Chakraborty and Picardal, 2013), implying that functional microorganisms are strongly dependent on the inoculum sources. The addition of lactate as a single organic substrate in this study may only stimulate lactate-utilizing microorganisms or lead to lactate-metabolism pathways, which may limit our findings as a result. However, the findings of the current study can still expand the microbial communities responsible for NO_3^- reduction and Fe(II) oxidation in paddy soil at circumneutral pH. It would be a good alternative to use the organic extract from the original soils as carbon source in future study, as this strategy could provide behaviors of NO_3^- reduction and Fe(II) oxidation in the presence of indigenous organic substrate. It should be noted that while no Fe(II) oxidation occurred in the treatment of Lactate + Fe(II), the addition of NO_3^- stimulated the Fe(II) oxidation with an average oxidation rate ± SD of 0.76 ± 0.043 mM d^{-1} in the treatment of Lactate + NO_3^- + Fe(II), suggesting that the presence of NO_3^- can make important contribution to the Fe(II) oxidation processes in the presence of carbon source in paddy soils. Though our results were obtained from the microcosm experiments, it could be proposed that the Fe(II) redox processes in the paddy soils with high Fe(II) contents from southern China can be strongly influenced by the NO_3^- reduction during the flooding season. The current study only reveals the kinetics of NO_3^- reduction and Fe(II) oxidation and the dominant microorganisms collected in the *in-vitro* culture. Field experiments will be needed in order to provide a real picture of the behaviors of NO_3^- reduction and Fe(II) oxidation as well as the associated microorganisms. Since high inputs and accumulation of organic C and organic N in paddy soils are induced by anthropogenic activities (Kögel-Knabner et al., 2010), further studies would be also valued if they could help understand how NO_3^--reducing Fe(II) oxidation influences the biogeochemical cycles of C and N, particularly C fixation and N loss (Ding et al., 2014).

4. Conclusions

NO_3^- reduction was completed within two days in the tested paddy soil suspension under anoxic conditions, and NO_2^- reduction was facilitated in the presence of Fe(II). However, only in the presence of NO_3^- could Fe(II) be oxidized. The phyla of *Proteobacteria* and *Firmicutes* dominated in the three treatments with lactate after amendment, but the relative abundance of each phylum varied with treatment. *Acidaminobacter*, *Proteiniclasticum*, *Alkaliphilus*, and *Natronincola_Anaerovirgula* dominated in the treatment with Lactate + NO_3^-, which expands the diversity of microbial communities associated with NO_3^- reduction. *Azospira*, *Zoogloea*, and *Dechloromonas* were found to be the dominant genera during NO_3^- reduction and Fe(II) oxidation, all of which reduce NO_3^- to nitrogen gas as the main end product rather than ammonium.

Acknowledgments

This work was funded by the National Natural Science Foundations of China (Nos. 41471216, 41330857 and 31400420), the Guangdong Natural Science Funds for Distinguished Young Scholar (2014A030306041), and the Australian Research Council DECRA grant (DE150100500). We also thank Dr. Weimin Yu in Guangdong Institute of Eco-Environmental and Soil Sciences for his assistance with soil classification.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.11.013.

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