Pyrosequencing revealed highly microbial phylogenetic diversity in ferromanganese nodules from farmland

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As unusual secondary minerals, ferromanganese nodules (FMNs) is an ideal materials in researching the Fe-Mn element biogeochemical cycling in nature. The previous studies of FMNs were focus on their mineral composition or structure. However, new evidence suggests that microorganisms play a significant role in the generation of FMNs. In this study, we used pyrosequencing to investigate the relative abundance, diversity, and composition of the microbial communities present in FMNs and their surrounding soil, which were collected from vertical soil profiles of both paddy fields and sugarcane fields. We are also sure that this article will not only provide a first-hand information of biogenetical origin for FMNs, but also generate a lot of interest for researchers involved in interaction between microbe-minerals.

Pyrosequencing revealed highly microbial phylogenetic diversity in ferromanganese nodules from farmland

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Abstract

There is a renewed interest in the origin and makeup of ferromanganese nodules (FMNs), long known to soil mineralogists as unusual secondary minerals. However, new evidence suggests that microorganisms play a significant role in the generation of FMNs. The biogenic origin of nodules has remained elusive because until recently, little was known about the overall microbial community structure in their microbiota. To learn more about the microbial community and to determine the relative abundance, diversity, and composition of the microbial communities present in FMNs and their surrounding soil, we used pyrosequencing to investigate 16S *rRNA* genes obtained from vertical soil profiles of both paddy fields and sugarcane fields. Using pyrotaq 16S *rRNA* gene sequencing, we show that the microbial phylogenetic diversity of nodules was higher than reported in previous studies of this biosphere, and we identified many previously unidentified microorganisms. Here, we show that the microbial community of these nodules is dominated by *Burkholderiales*, *Rhodocyclales*, *Acidobacteriales*, *Desulfuromonales,* and *Clostridiales*, and there were no statistically significant differences found in comparing the microbial community structures of FMNs obtained from vertical soil sequences. Although the microbial composition was markedly different between the surrounding soil and the FMNs, the microbes found within the FMNs were very similar to other FMNs from both field types examined here. In addition to their geochemical properties and the microbial community composition of FMNs, we found that the levels of iron (Fe), manganese (Mn), and SiO_2 greatly impact the microbial diversity among FMN communities. Our results indicate that the FMN microbial communities from different land-use types are very similar and suggest that natural selection of these microbes is based on the oligotrophic conditions and the high metal content. Researching FMNs in these two land-use patterns, which represent two different redox potentials, deepens our understanding of Fe-Mn biogeochemical cycling in these oligotrophic biospheres and suggests a biogenetical origin for these nodules.

Keywords: Ferromanganese nodules; microbial community structure; 16S *rRNA* gene

pyrosequencing; iron-manganese element biogeochemical cycling; paddy field; sugarcane field.

Abbreviations

FMNs: ferromanganese nodules;

qPCR: quantitative real-time PCR;

T-RFLP: terminal restriction fragment length polymorphism;

ARDRA: amplified ribosomal DNA restriction analysis;

DGGE: denaturing gradient gel electrophoresis;

OTUs: operational taxonomic units;

OM: organic matters;

Fe_d: dithionite extractable Fe;

Fe_o: oxalate extractable Fe;

Fe_p: pyrophosphate extractable Fe;

Mn_d: dithionite extractable Mn;

PCoA: principal coordinates analysis;

CCA: canonical correspondence analysis.

1. Introduction

The abundant transition metals, iron and manganese, are widely distributed in nature and actively participate in electron-exchange reactions in the environment.[1] Soil samples obtained from southern China often contain large quantities of iron and manganese, which undergo a rapid cycling between their reduced and oxidized states, which promote other element cycles in this environment.[2] These alternative redox cycles are driven by the diffusion of soluble Fe(II)/Mn(II) in a reducing environment, followed by the precipitation of new solid Fe(III)/Mn(IV) minerals in the oxygenated regions.[3] In addition, variations in oxygen diffusion and water content caused by differences in soil depth induce different redox potentials in the vertical horizon, which facilitate the Fe-Mn biogeochemical cycling in these soil profiles.[4]

Because of the ion exchange reactions that precipitate ore components from water, the ferromanganese nodules (FMNs) in the natural environment are often found at the boundary between the hydrosphere and the lithosphere (drying-wetting alternation region), where drastic oxidation and reduction cycles occur.[5] FMNs were first discovered on the ocean floor, but the plough soil such as paddy fields have a high level of anthropic activity, which provides a unique environment for the generation of FMNs. Here, the soil is consistently flooded during the main stages of rice growth and the fields are only left to drain after harvesting the rice.[6] Flooding fields results in soil reduction of plow layer, and the resultant Fe(II)/Mn(II) ions in the plow layer are leached by water percolation and accumulate in the subsoil depth, all of which are finally oxidized after the rice fields are drained.[7] Therefore, FMNs from plowed soils such as paddy fields and sugarcane fields provide a unique opportunity to probe the mechanisms behind Fe-Mn biogeochemical cycling in an artificial environment.

In nature, FMNs are widely distributed and have a high redox activity; FMNs are considered a critical model to elucidate the mechanisms of Fe-Mn element cycling in natural environments. Previous studies have focused on the mineralogy[8, 9] or geochemistry[10-13] of the FMNs. However, the unusual mineralogy of FMNs suggests they are not simply created by

the dissolution and leaching of iron/manganese minerals leaving insoluble materials behind. Many iron or manganese-oxidizing/reducing heterotrophic bacteria have been isolated from FMNs,[14-16] demonstrating that microorganisms play significant roles in FMN genesis.[17, 18] Previous researches suggest that a surprisingly phylogenettically diverse population of microorganisms are present on or inside the FMNs derived from terrestrial or aquatic biospheres.[6, 15, 19, 20] These pioneering studies demonstrate that *Acidobacteria*, *Proteobacteria*, *Firmicutes* and *Nitrospira* bacterium dominate the microbial phylotypes in FMNs.[19, 21] However, the rare archaeal biosphere of FMNs has not been explored in the previous studies, which mainly as a results of the low through-put analytical methods applied. The isolation of Fe- or Mn- depositing microorganism from the FMN environment also revealed that *Burkholderia* (e.g., *Leptothrix*) and *Bacillus* genus-affiliated bacteria broadly participate in the Fe-Mn biogeochemicals of such biospheres.[22] However, studies of the spatial distribution, diversity, and abundance of the microbial communities found in the FMNs derived from different land use patterns and soil profiles are still limited.

In this study, pyrosequencing 16S *rRNA* genes (targeting Bacteria and Archaea) were used to investigate the microbial phyla distribution in 13 FMNs-soil pair samples, which were taken from the vertical soil horizon in both a paddy field and a sugarcane field in an Fe-Mn rich region in China. Then, the difference in microbial diversity between FMNs and the associated surrounding soil was compared using several diversity indices. More specifically, by comparing the profiles of the microbial community structure and the pertinent element geochemistry of FMNs in the vertical soil sequences, we determined which abiotic factors influenced the abundance, diversity, and composition of microbial communities in FMNs. Combined with similar analyses of the associated surrounding soil, this study provides an overall view of the microbial composition found in FMNs, including rare microbes, which led us to further elucidate the microbial community members that are involved in the Fe-Mn biogeochemical cycling of soil.

2. Material and methods

2.1 Sample collection and the analysis of geochemical properties

Several kilograms of soil blocks, which contained red-brown FMNs, were collected from two sites located in Laibin City, Guangxi Province, south China during October 2011. One soil profile sample was taken from paddy soil (23°20'N, 109°31'E) with a 0-200 cm horizon depth and the other was from a sugarcane field (23°42'N, 109°38'E) with 0-100 cm vertical horizon sequences. The paddy soil and sugarcane filed samples used in this study are all belong to latosolic red soil, which are widely distributed in Guangxi province, China. There were 9 and 4 soil blocks samples collected from vertical horizons for analyses in both the paddy field and sugarcane field (20 cm depth interval). Totally, 26 samples (13 FMMs and 13 surrounding soil) were performed on the downstream geochemical and molecular analysis. Samples for biological analysis were collected into sterile 50 ml polypropylene tubes and shipped on ice to the laboratory where they were stored at -40 ° C. In the laboratory, approximately 15 g FMNs from sugarcane field (ca. 1 cm in diameter) and paddy soil (0.1-0.5 cm in diameter) (Supplementary Fig. S1), were carefully picked up with autoclaved tweezers and the soil attached to the nodules was separated. The nodules were immersed into distilled water and sonicated for 1 min to disperse clinging soil particles from the nodules. This washing step was repeated three times to obtain clean FMNs. The air-dried FMNs and surrounding soil were ground using a mortar and pestle until the entire sample passed through a 100-mesh sieve. The following geochemical characteristics were determined for each sample and used in the subsequent statistical analyses: pH, OM (organic matters), Fe_d (dithionite extractable), Fe_o (oxalate extractable), Fe_p (pyrophosphate extractable), Fe(II), Fe(III), total Fe, Mn_d (dithionite extractable), total Mn and weathering related materials (Fe_2O_3, SiO_2, Al_2O_3). The geochemical composition of the FMNs and the surrounding soil was determined as described previously.[2] The mineralogy of the FMNs was determined using powder X-ray diffraction (XRD) with a Bruker D8 diffractometer (Bruker Co., Ltd.) equipped with Cu X-ray tube operated at 40 kV and 40 mA. Scans were collected from 10° to 90° 2θ-angle, with a step size of 0.02° and a counting time of 1 s/step. Phase identification was executed by matching XRD patterns with

powder diffraction files (PDF) in the database published by the International Centre for Diffraction Data (ICDD).

2.2 DNA extraction from FMNs and the surrounding soil

Genomic DNA extraction from the FMNs and the surrounding soil was carried out, as previously described with slight modifications.[19, 23] Briefly, prior to genomic DNA extraction, the FMNs were intensively washed with distilled water and sterilized with a 0.1% NaClO solution for 1 min, rinsed three times with sterile distilled water, and then ground into a powder using a mortar and pestle. Then, genomic DNA was extracted from a 1.0 g sample of the ferromanganese powder using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. The DNA was eluted using 100 μL of TE buffer (10 mmol L^{-1} Tris-HCl (8.0), 1 mmol L^{-1} EDTA) and stored at -40 ° C. This genomic DNA extraction was performed in triplicate for each sample. The extracts were run on a 1% agarose gel and the quantity of the eluted DNA was estimated using the Qubit® dsDNA BR (Broad Range) Assay Kit (Invitrogen, Merelbeke, Belgium) in combination with a Qubit® 2.0 Fluorometer (Invitrogen, Merelbeke, Belgium).

2.3 16S rRNA PCR and 454 pyrotag sequencing

Analysis of the 16S rRNA genes was performed as previously described.[24] Briefly, the eubacterial sets of two primers 515F (5'- GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') were applied to amplify the 16S *rRNA* gene that was designed to amplify the hypervariable V3-V4 region of the 16S *rRNA* gene from nearly all bacteria and archaea.[25] The forward primer contained the Titanium A adaptor (5'-CGTATCGCCTCCCTCGCGCCATCAG-3') and the reverse primer contained the Titanium B adaptor (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3'). 8-bp Hamming barcodes that allow sample multiplexing during pyrosequencing were added to the 5' end of primer 515F. Three replicates of DNA extract from each sample were amplified by PCR. The

PCR was carried out with a PCR thermal cycler Model C1000 (Bio-rad Rad, Richmond, CA). The total volume of the reaction mixture was 50 μL containing 0.5 μL of each primer (50 pmol each), 5 μL of 2.5 mmol L^{-1} dNTP mixture, 5 μL of 10×*Ex Taq* buffer (20 mmolL^{-1} Mg^{2+}; TaKaRa Inc., Dalian, China), 0.25 μL of *Ex Taq* DNA polymerase (TaKaRa), 1 μL of environmental DNA template and 37.75 μL milli-Q water. Cycle conditions for the PCR amplification were as follows: initial denaturation at 94° C for 3 min, followed by 30 cycles of denaturation at 94 ° C for 30 s, annealing at 50 ° C for 30 s and extension at 72° C for 30 s, and 5 min at 72 ° C extension step after cycling was complete. All samples were amplified in triplicate and no template controls were included in all steps of the process. Next, 5 μl of each reaction mixture was analyzed on a 2% agarose gel and PCR products were gel-purified with a QIAquick Gel Extraction Kit (Qiagen). The purified PCR amplicons were combined in equimolar ratios into a single tube after the concentration of each amplicon was determined using the Quant-iT PicoGreen dsDNA reagent kit (Life Technologies, Merelbeke, Belgium) and sequenced by pyrosequencing using primer A on a 454 Life Sciences Genome Sequencer FLX (Roche Diagnostics, Indianapolis, IN, USA) machine following Titanium Chemistry at Macrogen Inc. (http://www.macrogen.com, Seoul, South Korea).

2.4 Bioinformatics analyses

Firstly, all 454 community 16S *rRNA* sequences were denoised (homopolymer error-correction) using Denoiser V0.91 software[26] according to the manual. Using QIIME-1.5.0[27], short reads were removed and only sequences longer than 200 bp were included in the downstream analysis, and low-quality reads were removed from the analysis using default settings (average quality value of > 20). Additionally, sequences containing > 6nt ambiguous nucleotides (“N”) in homopolymeric regions were removed. Sequences were then assigned to each sample by 8 bp barcode using a script derived from QIIME pipeline. Secondly, the remaining sequences from all of the samples were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity with “uclust” model (search and

clustering orders of magnitude faster than BLAST). The representative sequences in each OTUs were assigned to taxonomic groups using the RDP classifier[28] and comparing against SILVA database[29] within an 80% confidence threshold. Across all 13 nodule-soil pairs, we obtained 318,017 high quality sequences with an average read length of 285 bp after quality checking and denoising.[26] The number of sequences per sample ranged from 10,293 to 22,161, with an average of 13,827. Out of these sequences, 255,251 (80.26%) were classified below the phylum level with 70% confidence or greater, using the RDP classifier[28] and by comparing the sequences against SILVA database.[29] These sequences were affiliated with over 34 phyla across the entire data set. When we grouped sequences into operational taxonomic units (OTU) at the 3% dissimilarity level (roughly corresponding to the species' level), there were 36,018 OTUs in the complete dataset with an average of 1,762 OTUs per sample.

Lastly, to estimate alpha diversity, alignment of the sequences was conducted by PyNAST.[30] A random sub-sampling method from each sequence library was performed and used for microbial community diversity indices calculations to control for the effects of library size.[31] Alpha diversity indices (PD, Chao1 and observed species) analyses were performed for all samples with 100 repetitions using a step size of 5,000 sequences per sample. For beta diversity analysis, all samples were also subsampled to 5,000 sequences per sample to remove all possible side effects of sample size. Pairwise unweighted UniFrac distances calculated for the total community were analyzed in PRIMER v6.[32] The principal coordinates analysis (PCoA) was performed on pairwise unweighted UniFrac distances[33] using the QIIME software package. The DNA sequences were deposited in the MG-RAST database (http://metagenomics.anl.gov/)[34] with access numbers: 4502145.3

2.5 Statistical analyses

Correlation between microbial community diversity and environmental variables was estimated through Pearson correlation coefficients using the statistical software package SYSTAT 18 (SPSS, Inc.). Within each soil type, the divergence in microbial composition between ferromanganese nodules and surrounding soil was tested using the

independent-samples *t*-test. Correlations between community composition and environmental parameters were also analyzed by CCA (canonical correspondence analysis) using the program Canoco[35] and determine if the community structure is more strongly related to specific environmental variables than expected by chance.

3. Results

3.1 *The geochemistry of FMNs and their surrounding soil*

The geochemical characteristics of all 13 nodule-soil pairs (4 pairs from the sugarcane field and 9 pairs from the paddy field in two vertical soil profile) are presented in Table 1. The average pH value of sugarcane field was 4.87, while that of the paddy field was 6.39. Not surprisingly, the mean Fe_d (dithionite extractable), total Fe and Fe_2O_3 content of FMNs (average 228, 328 and 469 g Kg^{-1}, respectively for the sugarcane field; 184, 308 and 440 g Kg^{-1}, respectively for the paddy field) was significantly higher than in the associated soil (average 84.2, 89.2 and 127 g Kg^{-1}, respectively for the sugarcane field; 66.2, 86.1 and 123 g Kg^{-1}, respectively for the paddy field); the content of the oxalate extractable Fe, the pyrophosphate extractable Fe, Fe(II) and Fe(III) in both FMNs and their corresponding soils were in a similar range. The SiO_2 and Al_2O_3 content of FMNs (average 170 and 186 g Kg^{-1}, respectively for the sugarcane field; 133 and 204 g Kg^{-1}, respectively for the paddy field) were notably lower than the associated surrounding soils (average 367 and 276 g Kg^{-1}, respectively for the sugarcane field; 497 and 210 g Kg^{-1}, respectively for the paddy field). The total Mn was highly enriched in the FMNs (most were dithionite extractable Mn; average 0.17 g Kg^{-1} for the the sugarcane field and 1.87 g Kg^{-1} for the paddy fields) compared with the Mn in soil (average 0.08 g Kg^{-1} for the sugarcane field and 0.34 g Kg^{-1} for the paddy field). XRD studies revealed that the mineralogical makeup of these FMNs differs extensively between the sugarcane and paddy field (Supplementary Fig. S2): hematite [Fe_2O_3], kaolinite [$Al_2(OH)_4Si_2O_5$] and cronstedtite [$Fe_3FeSiO_4(OH)_5$] were the predominated minerals in the sugarcane field samples, while quartz [SiO_2], kaolinite, cronstedtite and goethite [α-FeO(OH)] were predominant in the paddy soil samples.

As shown in Fig. 1, the Fe and Mn found deep within the soil was more stable in FMNs than in the corresponding surrounding soil samples. Fe_d was mostly iron in the FMNs and the soil, but was found in larger concentrations deep in the soil of paddy fields. In addition, the content of Mn_d accounted for the vast majority of the total amount of Mn. The Fe(II)/Fe(III) ratio was 2.3 times larger in soil than in the associated FMNs from the sugarcane field, which indicated

a higher oxidation capability of the surrounding soil. However, the Fe(II)/Fe(III) ratio had an adverse pattern, indicating that the oxidation ability was stronger in FMNs than in the corresponding soil from the paddy field samples. The enrichment ratio[36] of weathering related materials in nodules from the paddy field and the sugarcane field exhibit similar patterns (Fig. 2), and showed the highest enrichment of Fe_2O_3 (~ 4), followed by Al_2O_3 and SiO_2 (< 1). Generally, the enrichment ratio of Fe_2O_3 and Al_2O_3 were decreased with the vertical soil depth in the paddy soil. However, data from the sugarcane field did not show such trends. The contrasting form and enrichment ratio of the Fe element in FMNs from these two samples, indicates that the iron cycle increased with vertical soil depth was more prominent in the paddy field than in the sugarcane field (Fig. 1 and Fig. 2).

As shown in Supplementary Table S1 and Supplementary Table S2, the correlation between geochemical properties exhibited different patterns in FMNs and the surrounding soil. Not surprisingly, the pH had a prominent correlation with other geochemical characteristics in the soil. The content of Fe_d and Mn_d also had a significant negative correlation in the surrounding soil (Pearson coefficient -.699, $P < 0.01$). Furthermore, the content of the weathering-related material had a significant correlation between the Fe- or Mn-content in the soil (See Supplementary Table S1 and Supplementary Table S2 for detailed). However, we did not detect a significant correlation between the geochemical parameters in the FMNs samples and the parameters from the surrounding soil. The most notable correlation was observed in the SiO_2 content with Fe_d (Pearson coefficient = .591, $P < 0.05$) and Mn_d (Pearson coefficient -.730, $P < 0.01$), which showed an adverse pattern in the surrounding soil (SiO_2 had a negative relationship with Fe_d and a positive relationship with Mn_d).

3.2 *The microbial diversity indices are affected by soil depth and geochemical characteristics*

We examined the microbial communities of FMNs and their associated soil at similar levels (5,000 randomly chosen 16S *rRNA* gene sequences per sample were conducted in our analyses). The results showed that in almost all nodule-soil pairs at an identical soil depth, the

predicted microbial community diversity (PD, Chao1 and observed species, at a genetic distance of 3%) in the surrounding soil exceeded that of the corresponding FMNs (Supplementary Table S3). However, the different microbial community diversity indices used (PD, Chao1 and observed species) did not show consistent trends between the FMNs and the surrounding soils with horizon depth, but the highest microbial indices were found at a depth of 20-40 cm in the sugarcane field sample and at a depth of 60-80 cm in the paddy field sample (Supplementary Table S3). Of those FMNs whose geochemical characteristics were considered, Fe_d and SiO_2 had a significantly positive correlation ($P < 0.01$) with either phylogenetic distance (PD) (Pearson coefficient 0.67 and 0.70 for Fe_d and SiO_2, respectively). But, there was a negative relationship observed between Mn content (Mn_d and total Mn) and species diversity indices (Chao1 and observed species) (Table 2). In the associated surrounding soil, we only detected that the Fe(II)/Fe(III) had a significantly positive correlation with PD (Pearson coefficient 0.60, $P < 0.05$) and Chao1(Pearson coefficient 0.87, $P < 0.01$) diversity index.

The average unweighted UniFrac distance[33] was computed (a measure of differences in microbial community structures) from OTU abundance patterns among all category pairs (including FMNs individuals with vertical soil depth in the identical site, nodule-soil pair in the same depth and FMNs from different sites) to access the microbial community differences in this system. To control for differences in coverage, all analyses were performed on an equal number of 5,000 randomly selected sequences. Overall, microbial community structure of FMN samples from the same soil vertical depth were much more similar to one another (average unweighted UniFrac distance 0.40 for paddy field samples and 0.33 for sugarcane field samples, respectively) than the FMNs samples from different sites (average unweighted UniFrac distance 0.42, respectively), while the surrounding soil from the vertical horizon had the most divergence in community structure (average unweighted UniFrac distance 0.70 for paddy field samples and 0.45 for sugarcane field samples, respectively) (Table 3). These results demonstrated that the microbiota inside the FMNs were stable in different land-use patterns and depth, although the surrounding soil exhibited large variance. As expected, PCoA

analysis (Principal Coordinates Analysis) using pairwise unweighted UniFrac distance showed that the FMN samples from the same land-use pattern clustered together, and that FMNs samples or associated soils from different location did not (Fig. 4). This suggested a phylogenetic dissimilarity between the microbial community structure of FMNs and surrounding soils in vertical soil depth rather than FMNs from different land-use patterns.

3.3 *The taxonomic compositions in FMNs and the corresponding surrounding soils*

Phylogenetic diverse eubacterial phylotypes were detected across all nodule-soil pair samples; β-*proteobacterial* sequences were most abundant (Fig. 3, Supplementary Table S4 and Supplementary Table S5). This phylum was dominated by members of *Burkholderiales* and *Rhodocyclales* (Supplementary Fig. S3). Most of the α-*proteobacterial* sequences were classified as *Rhizobiales* taxa while the *Pseudomonadales* taxon dominated the δ-*proteobacteria* phylum. The *Desulfuromonales* were the most abundant groups within the γ-*proteobacteria* phyla. Less predominant bacteria in FMNs and associated soils were *Acidobacteria*, *Antinobacteria*, *Verrucomicrobiae*, *Clostridia*, *Sphingobacteria*, *Cyanobacteria* (had a high abundance in topsoil of sugarcane field) and *Anaerolineae* (Supplementary Table S4 and Supplementary Table S5). The relative abundance of archaea was low both in FMNs (average of 1.78%) and surrounding soil (average of 3.36%). And about half of archaeal sequences (average of 46.5% for FMNs and 53.5% for surrounding soil) were been classified to unclassified archaea. Definitely rare biosphere, but still detected in this system (< 1%) included *Thermoprotei*, *Methanobacteria*, *Methanomicrobia*, *Thermoplasmata*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi* and *Nitrospira*. Comparative analysis of pyrosequencing data indicated that no significant differences were observed in the microbial composition in order level between the nodule-soil pairs from the same type of plough (Supplementary Fig. S3). Furthermore, the distribution of high abundance phyla exhibited no difference inside these two types of FMNs. But we identified several phyla (including β-*proteobacteria*, γ-*proteobacteria*, α-*proteobacteria*, δ-*proteobacteria*, *Cynaobacteria*, etc.) with significantly different levels of abundance in these two FMN

associated surrounding soils (Fig. 3).

3.4 *Correlations among selected geochemical properties and bacterial phyla distribution in the nodules-soils system*

The correlations between bacterial phyla and selected soil properties such as Fe_d, total Fe, Fe_o, Fe_p, Fe(II), Fe(III), Mn_d and total Mn were evaluated using ordination plots, according to the CCA (canonical correspondence analysis) (Fig. 5), in which the first two CCA axes explained 25.4%, 8.3% of the total variance in the FMNs microbial community data, comparing only explained 4.9%, 1.8% in the associated surrounding soils. CCA analyses showed, in FMNs biosphere, *Spingobacteriales* were centered along a gradient with relatively high Fe(II) or Fe (III) content. In contrast, *Acidobacteria* was associated with higher values of Mn_d and total Mn. However, the pattern in surrounding soils exhibited different trends, in that the total Fe and Fe_d contributed most on the microbial taxa distribution. Correlations between weathering related materials and bacterial phyla were revealed by the linear regression analyses (Supplementary Table S6). In FMNs, we found only *Mysococcales* had significant correlation with Fe_2O_3 (Pearson coefficient .629, $P < 0.05$) and SiO_2 (Pearson coefficient .700, $P < 0.01$), while in the associated surrounding soils, *Rhodocyclales* was associated with values of Fe_2O_3 (Pearson coefficient -.563, $P < 0.05$) and Al_2O_3 (Pearson coefficient .557, $P < 0.05$), but had a negative relationship with SiO_2 (Pearson coefficient -.703, $P < 0.01$).

4 Discussion

4.1 *Land-use patterns seriously impact on the microbial community structure of soil*

This investigation compared the difference between the microbial compositions, the diversity, and the geochemistry of soil from paddy fields and sugarcane fields, which are the primary land-use patterns of fields in south China. Paddy fields experience an alternation between reducing and oxidizing states during the stages of rice growth and harvesting. In contrast, sugarcane fields possess a higher oxygen content where the oxidative state remains dominated during crop growth. The Fe(II)/Fe(III) ratios of FMNs and the surrounding soil from the two types of land use studied here (Table 1), indicate that the oxidation capacity of sugarcane soil (mean Fe(II)/Fe(III) ratio 4.36) is much higher than that of paddy soil (mean Fe(II)/Fe(III) ratio 1.56). The higher the ratio of Fe(II)/Fe(III) in FMNs than that of the corresponding surrounding soil in paddy fields, suggests that Fe(II) oxidation occurs inside the nodules, while Fe(III) reduction occurs in the corresponding surrounding soil. The high oxygen levels and low pHs may be a response to the predominantly oxidized state of the sugarcane fields, while the relative low ratio of Fe(II)/Fe(III) within FMNs reveals that the reduction of iron is active on the surface of FMNs from sugarcane field. Previous research has demonstrated that the composition of the soil microbial community is closely correlated to land use patterns.[37] The relative high abundance of *Burkholderiales*, *Rhodocyclales*, *Acidobacteriales* and *Clostridiales* in these two types of plagues is in consistent with previous studies.[38, 39] The pH, oxidizing/reducing capacity (generated by different land use pattern) is responsible for the markedly different microbial community structures between paddy and sugarcane fields.

4.2 Iron- and manganese-redoxing bacteria

Microorganisms are active and may involve direct enzymatic interventions or the metabolic production of specific chemical reactants that cause precipitates to generate FMNs; this biogenic theory of FMNs is supported by the detection of respiring cells on the surface of FMNs using the in situ metabolic dye (2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride).[40] Nonetheless, by using

ultra-deep sequencing on the microbial community (> 10,000 sequences per sample), we successfully detected many iron- and manganese-reducing/oxidizing bacteria. Previous research using oxygenic gradient tube enrichments of circumneutral iron- or manganese-oxidizing bacteria from FMNs revealed functional groups including the *Rhizobiaceae* group in α-*Proteobacteria*, the *Actinobacteria*/*Bacillus* group in *Firmicutes* and the *Xanthomonas* group in the γ-*Proteobacteria*.[21] Our results demonstrate that these phylotypes are present in the FMNs and in the surrounding soils samples, but not the predominated species. The well-studied Fe-reducing anaerobic bacteria, *Geobacteraceae*[41] were recovered in large numbers using pyrosequencing data, and represented 1.1%-4.6% of FMNs, compared with 0.1%-4.5% in the surrounding soil (Supplementary Table S4 and Table S5). However, another facultative anaerobe, *Shewanellaceae*, that reduces iron and manganese metabolically,[42] was not detected in this large 16S *rRNA* sequence dataset. The relatively high abundance of *Geobacteraceae* suggests that this species is very involved in the Fe-Mn biogeochemical cycling in the FMN environment, and may reduce insoluble minerals such as iron- or manganese oxides and promote the mobility of the iron-manganese elements in the soil biosphere. In the presence of Fe(II), *Geobacter* is able to reduce hematite (the primary minerals in FMNs) better than *Shewanella* bacteria,[43] thus the physiology of *Geobacteraceae* is more suitable to the FMN habitat than *Shewanellaceae* in FMNs habitat.

Generally, microorganisms oxidize Mn(II) much faster than oxidation via abiotic means, suggesting that biological oxidation is dominant in nature,[44] and the manganese-oxidizing bacteria are actively functioning in the formation of FMNs. *Pseudomonas*, a genus which contains well-studied manganese-oxidizing species,[45] is relatively abundant in our data, indicating its importance in manganese biogeochemical cycles of microbial ecosystem within the FMNs. *Leptothrix* belong to *Burkholderiales* and are known for their oxidation of both Fe(II) and Mn(II) in their sheath.[46] In this study *Leptothrix* was present in 0.7%-2.9% of the total 16S *rRNA* sequences from the FMNs. Another iron-oxidizing chemolithotrophic bacterium, *Gallionela*, oxidizes Fe(II) to Fe (III) for energy conservation,[47] but was not detected in the FMN microbial community in this study. All of these data indicate that

Geobacteraceae and *Leptothrix* play important roles in FMNs mineral formation during the process of biological precipitation or the dissolution of Fe-Mn elements.

4.3 The impact of minerals weathering on the FMN microbial communities

Increasing evidence suggests that microbes play a significant role in mediating the dissolution and mineral oxidation, which modifies the rate and mechanism of chemical and physical weathering and clay growth.[48, 49] Fe(II) is produced on the subsurface under anoxic conditions by dissimilatory iron(III) reducing bacteria (DIRB) coupled with biotic/abiotic weathering of minerals.[50] Herein, we observed the relationship between weathering minerals (Fe_2O_3, SiO_2, Al_2O_3), microbial diversity, and the composition to investigate the link between the weathering process and the presence of certain microbial populations. Researching the subsurface environments has revealed that silicate weathering caused by bacteria is dependent on the trace nutrient contents of each aquifer mineral.[51] Our results show that the SiO_2 content has a significantly positive correlation with microbial diversity within FMNs (PD diversity, Pearson coefficient .702, $P < 0.01$), but the associated surrounding soil did not exhibit such patterns (Table 2). Silicon, a beneficial nutrient, can limit microbial growth and metabolism.[51] In the nutrient-limited FMN biosphere, the progression of weathering may be influenced by SiO_2 nutritional potential, which stimulates microorganisms to accelerate the dissolution of clay minerals and enhance the release of SiO_2 and other nutrients from FMN minerals, ultimately increasing the diversity of the microbial community. These results support the idea that microbial activity is a major driving force behind mineral weathering even in the isolated environment of FMNs. The biochemistry of SiO_2 utilization by microbes is not well understood.[17] However, the significant positive correlation between the SiO_2 content and the presence of *Myxococcales* bacterium (Supplementary Table S2) suggest that these bacteria may participate in silicon's biogeochemical cycling in FMN's ecosystem. Furthermore, a dissimilatory iron-reducing member of the *Anaeromyxobacter* subgroup of *Myxococcales* has been isolated,[52] demonstrating the importance of these bacteria in Fe-Si coupled element cycling in nature and in the process of nodule formation.

4.4 The microbial community structure is stable in FMNs from different land-use patterns and from vertical soil profiles

The sugarcane soil had a lower pH (average pH 4.9) than the paddy soil (average pH 6.4), which accounts for the high divergence of microbial compositions in the FMNs associated with the surrounding soil from both sugarcane and paddy fields (average unweighted Unifrac distance 0.54), which coincides with the idea that pH has a significantly influence on the diversity of soil microorganisms.[53-55] Likewise, the pH of the soil in the vertical horizon in paddy fields (from 4.81 to 7.97) was responsible for the marked discrepancy between the microbial community structure in surrounding soil and the content from different vertical horizon depths (average unweighted Unifrac distance 0.70). Although the physical and chemical properties of FMNs from different soil profiles has been investigated,[56-58] the microbial community of nodules in vertical soil depth have only been addressed by Cahyani et al., revealed that the bacterial communities in the manganese nodules were markedly different from those in plowed layer of rice field and their subsoil.[6] Our study demonstrated that the community structures were significantly different in the FMNs associated with the surrounding soil from these two types of plowed fields or in vertical soil sequences, however, the microbiota in FMNs were very similar despite the different land-use patterns (average unweighted Unifrac distance 0.42, Table 3) or the different soil depths (average unweighted Unifrac distance 0.40 and 0.33 for paddy field and sugarcane field samples, respectively). This disequilibrium suggests that the ecological and evolutionary forces behind the development of FMNs (typical high metals content and low organic matters content, Table 1) may shape their microbial diversity and community structure in this oligotrophic and isolated environment, which results in a particular structure of microbial composition. Without available carbon and nitrogen sources, FMN microbes derive energy mainly by transferring electrons between iron and manganese oxides and hydroxides, to sustain the reproduction of microorganisms. Due to this “function-selection”,[59] the microbial community structure is stable in FMNs regardless of the community divergence observed in their surrounding soil

biogeochemical properties. The dispersal limitation primarily driver the biogeographical patterns in soil, and the geographic distance should be the best predictor of genetic divergence between communities and habitats.[60, 61] This is not the case in the FMN environment; the geographic factors that determine the spatial pattern of microbial communities were not demonstrated in our analyses, and can only be evaluated in further studies on more systematical samples and record data from various distances and soil types.

Conclusion

We used pyrosequencing to analyze the microbial communities present in ferromanganese nodules, which were collected from vertical soil profiles of paddy and sugarcane fields. We found high phylogenetic diversity among the microbes from these oligotrophic biospheres, including many phylotypes, which have not been reported previously. Our studies also suggest the presence of an iron- and manganese-oxidizing or reducing microorganism in the FMN environment, and *Geobacteraceae* and *Leptothrix* are the major functional groups active in Fe-Mn biochemical cycling. Biomineralization plays an important role in secondary mineral and soil formation, and weathering-related components (such as Fe_2O_3, SiO_2 and Al_2O_3) determined the degree and extent of weathering. Our study assessed the correlation between weathering indices and the diversity of the microbial community or the taxonomic composition, and found that SiO_2 content was correlated with increased microbial diversity in the FMN environment, which increases our understanding of the role that microorganisms play in the weathering process. Only certain organisms can survive in the presence of oligotrophic forces and a high-metal environment; the natural selection of organisms that thrive in this environment can explain the similar microbial communities we observed in FMNs from different land-use patterns and vertical soil sequences.

Currently, many researchers are dedicated to the isolation, characterization, and quantification of iron- and manganese-oxidizing bacteria from the environment. Other studies are focused on determining the rates of iron and manganese oxidation and on finding key species or genes that respond to this process using genomic, transcriptomic, and proteomic methods. This

research elucidates the significant role that microbes play in the mechanism and generation of ferromanganese nodules. However, to determine the biogenic mechanisms of FMNs in natural ecosystems, further studies using metagenomic and metranscriptomic techniques in combination with iron- and manganese-isotope fractionation and systematic sampling are required. Further studies are also necessary to investigate the genes or species that respond to the Fe-Mn biochemical cycling in these environment to ultimately answer the question: "How do biotic factors impact the production of these unusual secondary minerals?"

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Tables Captions

Table 1. Physicochemical characteristics of the ferromanganese nodules and surrounding soil samples.

Table 2. Pearson correlations of values of soil physicochemical properties with microbial alpha diversity indices.

Table 3. Unweighted uniFrac distance between samples.

Table 1. Physicochemical characteristics of the ferromanganese nodules (A) and surrounding (B) [a]

(A)

Samples	Sugarcane FMNs						Paddy FMNs					
	Mean	SD	Median	Min	Max	CV	Mean	SD	Median	Min	Max	CV
pH	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
OM (g/Kg)	2.68	0.22	2.72	2.39	2.89	0.08	**3.56***	0.53	3.67	2.26	4.03	0.15
Fe_d (g/Kg)	**228***	20.2	229	203	252	0.09	**184***	21.6	186	138	209	0.12
Fe_o (g/Kg)	8.07	0.15	0.89	0.69	1.02	0.18	0.83	0.21	0.84	0.58	1.14	0.25
Fe_p (g/Kg)	0.19	0.04	0.20	0.14	0.23	0.22	0.22	0.10	0.19	0.15	0.49	0.48
Fe(II)(g/kg)	0.10	0.02	0.09	0.09	0.12	0.16	0.11	0.04	0.10	0.05	0.19	0.37
Fe(III)(g/kg)	0.06	0.02	0.05	0.04	0.09	0.42	0.04	0.03	0.04	0.01	0.11	0.72
Fe(II)/Fe(III) (g/Kg)	1.86	0.64	2.14	0.91	2.26	0.34	3.7	2.95	3.32	0.95	10.83	0.80
Total Fe (g/Kg)	**328***	20.1	335	299	344	0.06	**308***	15.0	311	275	323	0.05
Mn_d (g/Kg)	0.14	0.06	0.14	0.07	0.22	0.45	**1.42***	0.51	1.25	0.90	2.31	0.36
Total Mn (g/Kg)	0.17	0.06	0.14	0.07	0.21	0.40	**1.87***	0.46	1.74	1.42	2.76	0.24
Fe_2O_3 (g/kg)	**469***	28.7	478	428	492	0.06	**440***	21.4	444	393	462	0.05
SiO_2 (g/kg)	**170***	5.60	169	165	178	0.03	**133***	7	136	121	144	0.05
Al_2O_3 (g/kg)	**186***	18.3	184	171	207	0.10	**204***	11.2	203	190	225	0.05
SiO_2/Al_2O_3	**0.92***	0.08	0.93	0.81	0.99	0.09	**0.66***	0.05	0.65	0.60	0.74	0.08
Al_2O_3/Fe_2O_3	**0.4***	0.06	0.39	0.35	0.48	0.16	**0.46***	0.03	0.45	0.43	0.52	0.06
$SiO_2/(Al_2O_3+Fe_2O_3)$	**0.26***	0.01	0.26	0.25	0.27	0.03	**0.21***	0.02	0.21	0.19	0.23	0.07

(B)

Samples	Sugarcane SS						Paddy SS					
	Mean	SD	Median	Min	Max	CV	Mean	SD	Median	Min	Max	CV
pH	4.87	0.53	4.645	4.53	5.67	0.11	6.39	1.58	7.05	4.45	8.22	0.25
OM (g/Kg)	11.7	8.09	8.89	5.99	23.2	0.69	**9.41***	3.87	8.11	5.71	18.9	0.41
Fe_d (g/Kg)	**84.2***	6.06	86.7	75.2	88.3	0.07	**66.1***	9.66	67.4	55.5	80.1	0.15
Fe_o (g/Kg)	0.80	0.58	0.55	0.45	1.67	0.73	1.11	0.49	0.98	0.59	1.89	0.44
Fe_p (g/Kg)	0.11	0.11	0.07	0.04	0.27	0.97	0.17	0.12	0.13	0.08	0.45	0.71
Fe(II)(g/kg)	0.09	0.04	0.08	0.06	0.15	0.44	0.11	0.06	0.09	0.07	0.26	0.52
Fe(III)(g/kg)	0.02	0.01	0.02	0.01	0.04	0.57	0.11	0.12	0.06	0.02	0.40	1.10
Fe(II)/Fe(III) (g/Kg)	4.36	1.60	4.43	2.39	6.20	0.37	1.56	0.78	1.41	0.65	3.23	0.50
Total Fe (g/Kg)	**89.1***	5.03	90.6	82	93.5	0.06	**86.1***	6.50	86.1	74.9	96.8	0.08
Mn_d (g/Kg)	0.11	0.03	0.10	0.08	0.15	0.32	**0.24***	0.09	0.20	0.17	0.47	0.39
Total Mn (g/Kg)	0.08	0.02	0.07	0.06	0.11	0.29	**0.34***	0.09	0.21	0.13	0.43	0.37
Fe_2O_3 (g/kg)	**127***	7.19	129	117	134	0.05	**123***	9.29	123	107	138	0.08
SiO_2 (g/kg)	**367***	11	366	358	381	0.03	**497***	59.3	517	430	590	0.10
Al_2O_3 (g/kg)	**276***	24.1	282	246	297	0.09	**209***	38.8	193	139	239	0.17
SiO_2/Al_2O_3	**1.3***	0.10	1.31	1.25	1.46	0.07	**2.49***	0.79	2.67	1.80	4.24	0.30
Al_2O_3/Fe_2O_3	**2.2***	0.20	2.24	1.91	2.30	0.08	**1.79***	0.29	1.79	1.13	1.87	0.16
$SiO_2/(Al_2O_3+Fe_2O_3)$	**0.91***	0.04	0.91	0.86	0.96	0.04	**1.53***	0.37	1.69	1.16	2.25	0.22

[a] FMNs: ferromanganese nodules; SS: surrounding soil; OM: oganic matter; Fe_d: dithionite extractable Fe; Fe_o: oxalate extractable Fe; Fe_p: pyrophosphate extractable Fe; Mn_d: dithionite extractable Mn; NA: not available. Asterisk represented significantly different ($P < 0.05$) between ferromanganese nodules and corresponding surrounding soil examined by independent *t*-test. The value of physicochemical characteristics was showed as Mean, SD, Median, Min , Max and CV in sugarcane field samples (N=4) and paddy field samples (N=9) from vertical soil profile, respectively.

Table 2. Pearson correlations of values of soil physicochemical properties with microbial alpha diversity indices [a].

	Ferromanganese nodules			Surrounding soil		
	PD*	chao1	Observed species	PD	chao1	Observed species
pH	NP	NP	NP	.280	.497	.035
OM	-.42	-.519	-.430	-.240	.137	.017
Fe_d	**.673***	**.696****	**.635***	-.307	-.369	-.083
Fe_o	.242	.347	.305	.057	.271	.007
Fe_p	-.339	-.286	-.328	.121	.234	.095
Fe(II)	-.178	-.094	-.124	.092	.284	.125
Fe(III)	.073	.145	.090	.241	.381	.250
Fe(II)/Fe(III)	-.121	**-.615***	-.199	**-.595***	**-.887****	-.384
Total Fe	.341	.399	.408	-.341	-.446	-.074
Mn_d	-.371	**-.587***	-.460	.155	.214	.054
Total Mn	-.511	**-.714****	**-.584***	.164	.231	.190
Fe_2O_3	.341	.399	.408	-.341	-.446	-.074
SiO_2	**.702****	**.890****	**.767***	.160	.275	-.052
Al_2O_3	.017	.242	-.052	-.090	-.248	.111
SiO_2/Al_2O_3	.501	**.736****	**.577***	.229	.393	.059
Al_2O_3/Fe_2O_3	-.149	-.353	-.228	.008	-.129	.129
$SiO_2/(Al_2O_3+Fe_2O_3)$	**.596***	**.815****	**.653****	.225	.383	.020

[a] PD: phylogenetic distance. Marked correlations are significant at 0.05 (*) and 0.01 (**) level of probability. NP: not possible to calculate.

Table 3. Unweighted Unifrac distance between samples [a].

	Paddy FMNs	Paddy SS	Sugarcane FMNs	Sugarcane SS
Paddy FMNs	0.40 ± 0.11			
Paddy SS	0.59 ± 0.23	0.70 ± 0.24		
Sugarcane FMNs	0.42 ± 0.09	0.58 ± 0.24	0.33 ± 0.05	
Sugarcane SS	0.51 ± 0.16	0.54 ± 0.21	0.44 ± 0.16	0.45 ± 0.17

[a] Mean (±SD) unweighted UniFrac distance for pair-wise comparisons between FMN individuals with soil depth in identical place, nodule-soil pair in the same depth and FMNs from different sites. Samples designated were included in Table 1.

Figure Captions

Fig. 1. Key iron and manganese profile characteristics in soil depth profiles.

Fig. 2. Plot of enrichment factors for major weathering materials with soil depth profile.

Fig. 3. Relative abundance of selected microbial taxa in the ferromanganese nodules and surrounding soil samples.

Fig. 4. Principal component analysis (PCoA) derived from pairwise unweighted UniFrac distances between microbial communities of ferromanganese nodules and surrounding soil.

Fig. 5. Canonical correspondence analysis (CCA) relating 16S *rRNA* gene sequence patterns with Fe-Mn element variables and microbial characteristics in different ferromanganese nodules and surrounding soil.

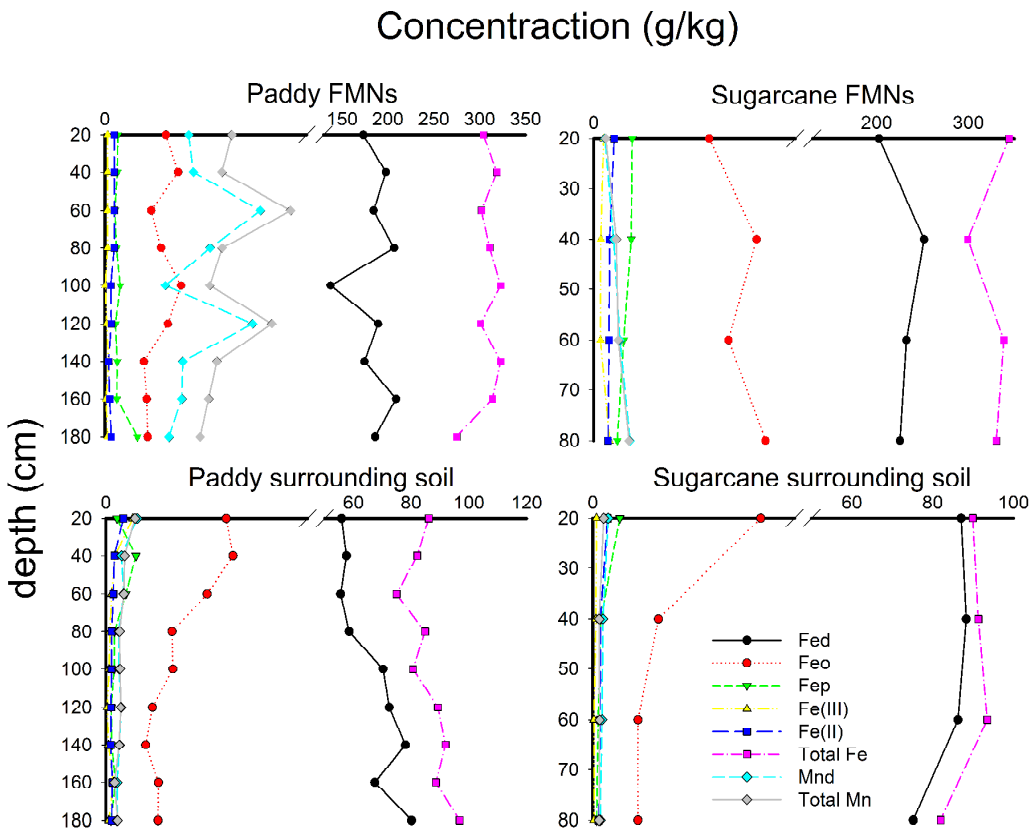

Fig. 1. Key iron and manganese profile characteristics in vertical soil depth sequences. Sample designation refers to Table 1. Complete physicochemical characteristics are available in Table 1.

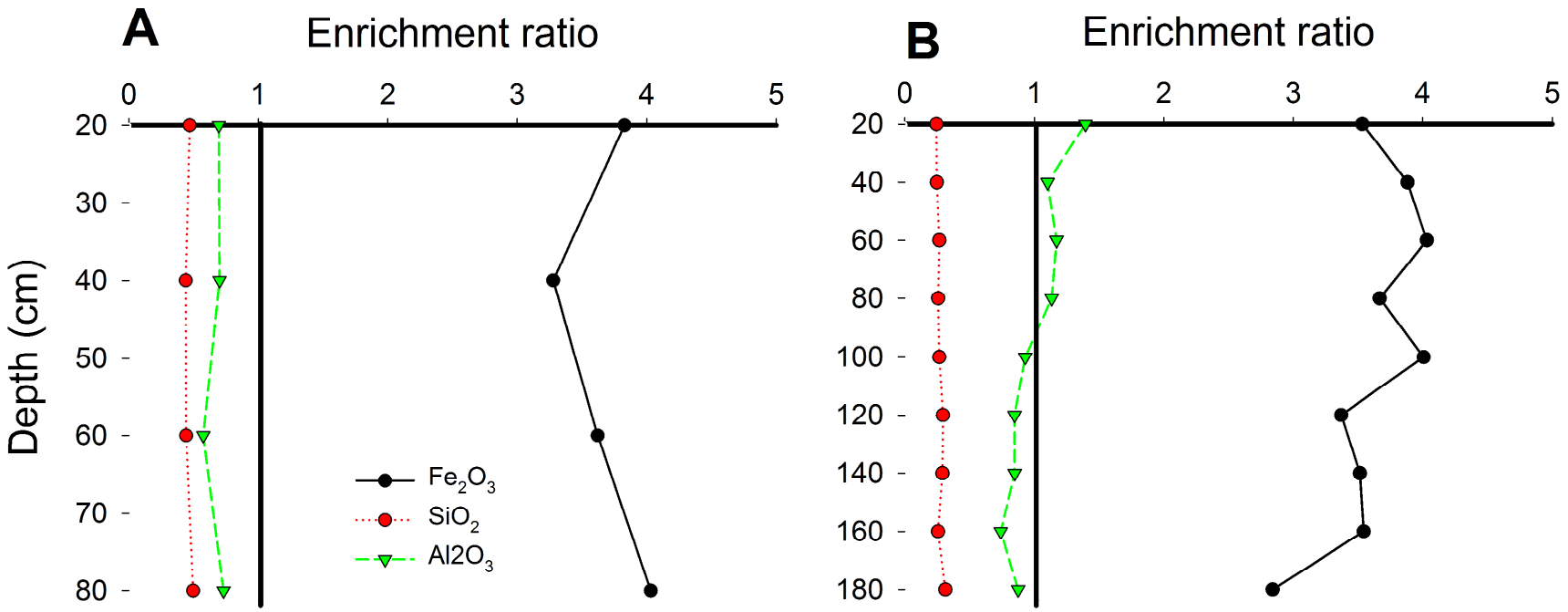

Fig. 2. Plot of enrichment factors for major weathering materials with soil depth profile. Line plot showing enrichment ratio of weathering related materials in FMNs compared to soils with vertical depth profile in sugarcane (A) and paddy (B) field samples. The enrichment ratio is the concentration of the materials in the ferromanganese nodules divided by the concentration of the materials in the associated surrounding soil.[36] Data was the same as that in Table 1.

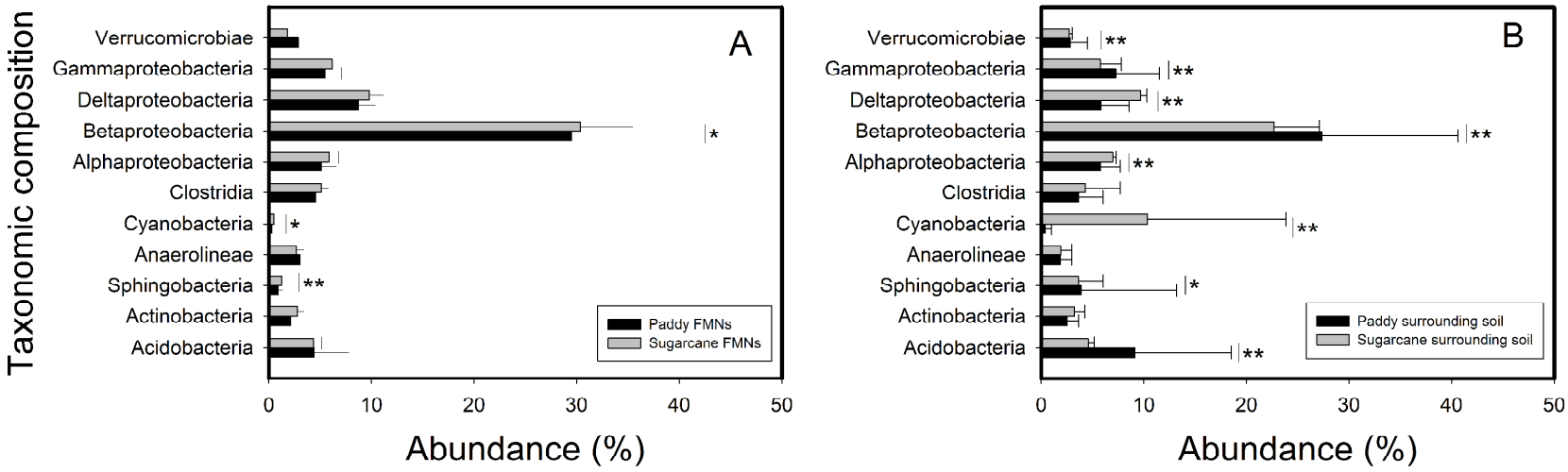

Fig.3. Relative abundance of selected microbial taxa in ferromanganese nodules (A) and surrounding soil (B) samples. The value were showed as mean ± SD in sugarcane field samples (N=4) and in paddy field samples (N=9). Asterisk represented significantly different ($^{*}P < 0.1$; $^{**}P < 0.05$) between samples from paddy field and sugarcane field examined by independent *t*-test. Microbial species with relative abundance higher than 1% were shown.FMNs: ferromanganese nodules.

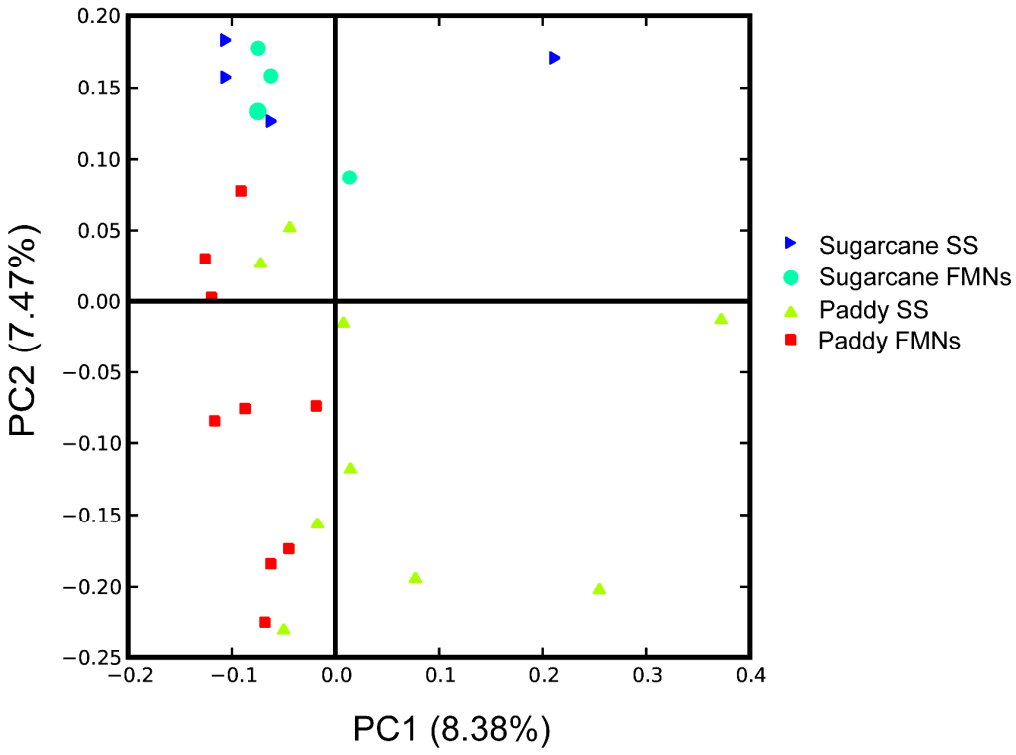

Fig.4. Principal component analysis (PCoA) derived from pairwise unweighted UniFrac distances of 16S rRNA gene between microbial communities of ferromanganese nodules and surrounding soil. The analysis was performed on a randomly selected subset of 5,000 sequences per sample. Values in parentheses on the axis labels indicate the percentage variation accounted for by each axis. FMNs: ferromanganese nodules; SS: surrounding soils.

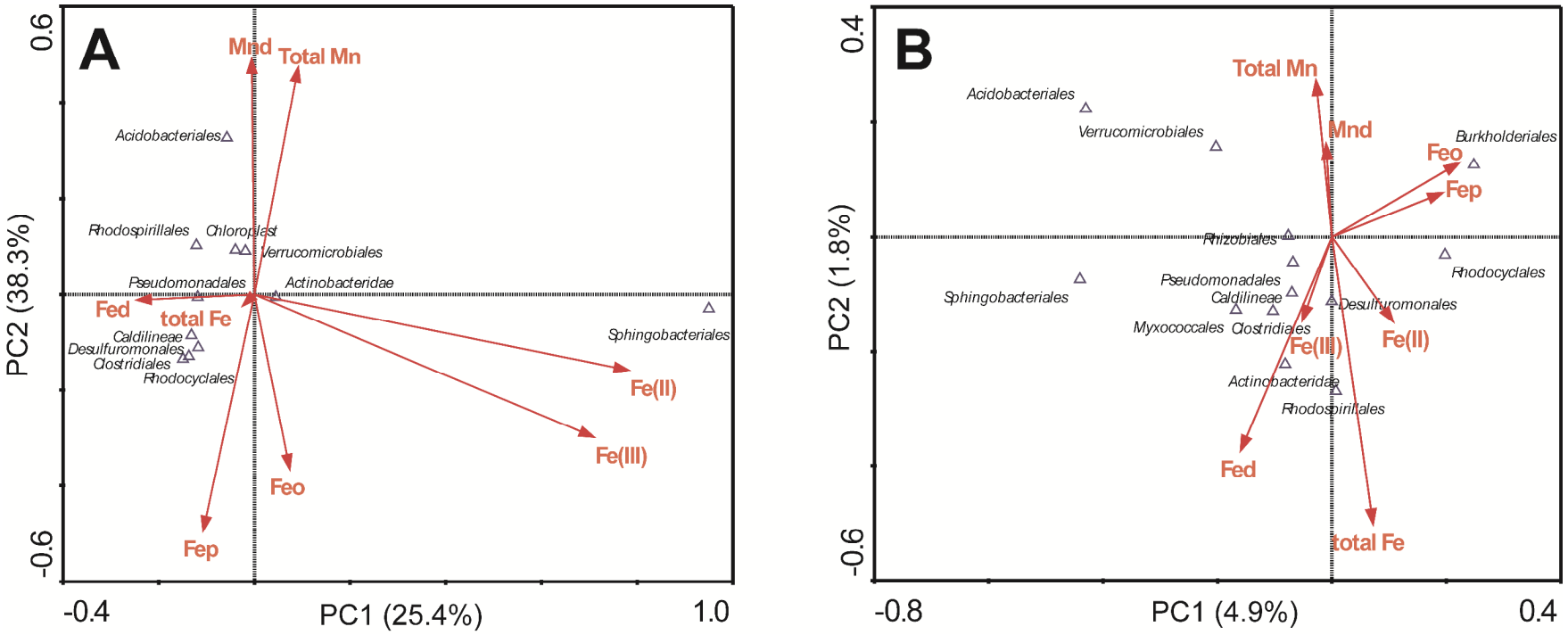

Fig. 5. Canonical correspondence analysis (CCA) relating 16S rRNA gene sequence patterns with Fe-Mn element variables and microbial characteristics in different ferromanganese nodules (A) and surrounding soil (B). Relating environmental variables were showed as red arrows, while microbial groups were showed as black hollow triangle. Values in parentheses on the axis labels indicate the percentage variation accounted for by each axis