African Journal of Agricultural Research Vol. 6(28), pp. 6107-6118, 26 November 2011
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.1159
ISSN 1991-637X ©2011 Academic Journals

Full Length Research Paper

Variation of *Anaeromyxobacter* community structure and abundance in paddy soil slurry over flooding time

Chao Zhu[1], Shu-Hong Xia[1], Bao-li Wang[1] and Dong Qu[2]*

[1]College of Life Science, Northwest A and F University, 3 Taicheng Road, Yangling 712100, Shaanxi, China.
[2]College of Resources and Environment, Northwest A and F University, 3 Taicheng Road, Yangling 712100, Shaanxi, China.

Accepted 19 September, 2011

Cultivation-independent techniques like PCR-amplified restriction fragment length polymorphism (PCR-RFLP) of 16S rRNA genes and real-time PCR were applied to assess the abundance, diversity and phylogenetic composition of *Anaeromyxobacter* communities over time in flooded, unplanted paddy soil slurries. Six *Anaeromyxobacter* communities were sampled from anoxic slurries at 1 h, and 1, 5, 10, 20 and 30 days while the Fe (II) concentrations were measured also. Bacterial Genomic DNA was extracted and PCR-amplified to obtain 16S rDNA fragments of *Anaeromyxobacter* which were cloned to construct 6 16s rDNA libraries. Eventually 10 major *Anaeromyxobacter* types were identified by RFLP fingerprintings. Results showed that the optimal increasing phase of Fe (II) was from 1h to nearly 10 days, being correspond with the growth phase of the abundance of *Anaeromyxobacter*. The highest diversity appeared in slurry at 30 days and the lowest was found at 30 days. Jackknife Environment Clusters by UniFrac showed that phylogenetic compositions of *Anaeromyxobacter* communities in slurries at 10 and 20 days were the most similar. By evolutionary distance analysis, our 10 major *Anaeromyxobacter* types were diverged into Group 1 and 2 in phylogenetic tree, while Group 1 was the exclusive collection of clones from our experiment. Major type P1 was present in all slurries abundantly and P9 only existed in slurry at 5 days. The abundance of *Anaeromyxobacter* spp., calculated as its proportion of 16S rDNA copies to the value of total *Bacteria*, was from 0.242% at 1 h to 5.135% at 10 days. We demonstrated that flooding time led to successional dynamics of major types and variable abundance of *Anaeromyxobacter* community. Flooding time also influenced the diversity of *Anaeromyxobacter* community on some extent. Canonical correspondence analysis (CCA) revealed that *Anaeromyxobacteria* spp. abundance had interrelation with Fe (II) content and the influenced the distribution of the slurries in the Biplot. Moreover, our study provides valuable information for the further isolation of *Anaeromyxobacter* strains from paddies.

Key words: *Anaeromyxobacter*; paddy soil, community structure, abundance, PCR-RFLP, flooding time.

INTRODUCTION

Anaeromyxobacter spp., as gram-negative bacteria, is considered to be a unique member of the order of Myxococcales (mycobacteria). *Anaeromyxobacter* species were found to be process anaerobic and microaerophilic lifestyles, whereas all other identified mycobacteria were characterized as obligate microbes. Being able to deriving energy from dissimilatory Fe (III) reduction (Sanford et al., 2002; He and Sanford, 2003), *Anaeromyxobacter* species also are of great anaerobic respiratory versatility by coupling the oxidation of acetate or hydrogen and the reduction of a wide range of compounds such as organic halide, soluble and amorphous, nitrite, nitrate, fumarate, humus, and oxygen (He and Sanford, 2002). All these physiological properties allow *Anaeromyxobacter* spp. to be considered as the microorganism being capable of bioremediation in the contaminations of halogenated compounds (He and Sanford, 2002 and 2003; Thomas et al., 2009), U (VI) (Wu et al., 2006; Sanford et al., 2007; Marshall et al.,

*Corresponding author. E-mail: dongqu@nwsuaf.edu.cn or 304545693@qq.com. Tel: +86-(0)29-87082624.

2009; Thomas et al., 2010), Tc (VII) (Marshall et al., 2009) and Se (IV) (He and Yao, 2010) in the sedimentary environment, whereas the full mechanism of these processes and the exploitation and domestication of engineering *Anaeromyxobacter* strain for practical utilization still require further study.

Various uncultured and few cultured members of *Anaeromyxobacter* were found in contaminated sediments, garden compost, and estuarine sediments (Cole et al., 1994; Wu et al., 2006; Lin et al., 2007; Thomas et al., 2009;). However, very few reports focused on this genus in flooding paddy soil, where anaerobic Fe (III) reduction acts as the second most important electron sink (Yao et al., 1999) and thus would be an ideal habitat for *Anaeromyxobacter*. To date, few *Anaeromyxobacter* strains like *Anaeromyxobacter* sp. FAc12, an iron reducing bacteria also as an acetate oxidizer, was isolated, revealing the existence of more uncultured *Anaeromyxobacter* spp. around the rice rhizosphere (Treude et al., 2003). Furthermore, Hori et al. (2007) indicates *Anaeromyxobacter* spp., which can outcompete with methanogen for acetate in low concentration, is one of the dominant Fe (III) reducers in paddy soil amended with goethite. Flooding has been confirmed to have obvious effects on the soil properties and thus its microbial communities such as the decrease of microbial biomass, aerobic bacterial, gram-negative bacteria and gram-positive bacteria markers (Reichardt et al., 2001; Gelsomino et al., 2006; Unger et al., 2009). Lüdemann (2000) and Noll (2005) described the spatial and temporal shifts in the bacterial community structure in flooded paddy soil, respectively. But no information is available on the community structure of *Anaeromyxobacter* species and their successional dynamics in flooding paddy soil, as well as their abundance variation. As a microbial species play potential roles in the Fe (III) reduction and the degradation of possible contaminant in paddy soils, more knowledge about *Anaeromyxobacter* spp. are still require for exploration.

As an effort to extend our knowledge about the environmental function of the *Anaeromyxobacter* spp. in paddy soil, we visited the anaerobic paddy soil slurry in different flooding stages, tried to elucidate the following questions: How does *Anaeromyxobacter* community and abundance change over flooding time or will they shift a lot? Can the slurries of different time reflect some expected clustering among themselves based on the environmental factors? What is the correlation between *Anaeromyxobacter* spp. and microbial Fe (III) reduction in flooding paddy soil? Also, these answers may benefit us for the further isolation and identification of *Anaeromyxobacter* spp. from flooding paddy soil. In this study, we adopted 16S rDNA gene as an operational taxonomic unit (OUT) (Sjoling and Cowan, 2003) to analyze microbial community, while RFLP (Sergio et al., 2004) patterns which generated by restriction enzyme digestion and sequencing information were employed to perform bacterial clone library analysis (Haddad et al., 1995; Oliver et al., 2005). The fingerprints visualized the composition of the *Anaeromyxobacter* community, allowing for a rapid comparison of samples varying over time (Michael et al., 2003). We also designed and applied an real-time PCR approach to characterize and monitor the *Anaeromyxobacter* abundance in flooding incubations of different times.

MATERIALS AND METHODS

Soil sampling

Soil was taken from drained paddy field after harvesting, Longtanduncun in Fangqiao (29°46′N, 121°27′E), located in Fenghua, Zhejiang, which belongs to the double cropping area in southeast China. Soil sample was collected from the top 20 cm, removed the plant residual, air-dried, ground, and passed through a 2 mm sieve. Considering reproducibility issues and biotic error, we sampled at 7 different places in the field, and pooled soil cores for subsequent incubation. Soil sample was kept in polyvinylchloride bottles at room temperature. The organic matter content in the soil sample was 54.7 g kg^{-1}, the pH was 5.5 (in 5 parts H_2O to 1 part soil), amorphous iron concentration was 6.2 g kg^{-1}, and free iron concentration was 10.4 g kg^{-1}.

Slurry incubation and Fe (II) measurement

Three gram soil was mixed with sterilized deionized water at a ratio of 1:1 (wt / vol) in a 10 ml sterilized serum bottle. Oxygen was removed by gassing nitrogen into the bottle at a ratio of 2 L/min (1.01×10^5 Pa) for 1 min. All the bottles were sealed with butyl rubber cover and aluminum cap and then incubated at 30℃ in the dark. Three microcosms for each treatment were homogenized and destructively sampled. Samples for the immediate Fe(II) measurement were taken from triplicate slurry incubations after flooding of 1 h and 1, 5, 10, 20, and 30 days, while another 3 samples were simultaneously sampled, pooled and stored under -20℃ for the molecular analysis. The determination o f Fe (II) content and Logistic and the simulation of the relationship between Fe (II) accumulation and incubation time in each treatment were using the standard procedure (He and Qu, 2008).

Extraction of total DNA

DNA was directly extracted from 3 g paddy soil slurry by the protocol of Zhou (1996) with a few modifications. Every time 0.3 g soil slurry was ground with the presence of liquid nitrogen and the produce was transferred to clean tube for DNA extraction. This process was repeated for 10 times and all the extracted DNA were mixed together. PCR inhibitors like humid acid in the raw DNA extraction was separated by electrophoresis using 0.8% agarose gel under 70 V for 30 min. Purified DNA was then recycled from the gel by TIANgel Midi Purification Kit (TIANGEN Biotech Co. Ltd., Beijing).

Construction of 16S rRNA gene clone library

Anaeromyxobacter spp. specific primers FAc12-66F (5'-CGA GAA AGC CCG CAA GGG-3') and FAc12-432R (5'-CGT CCC TCG CGA CAG TGC-3') (friendly offered by Prof. Schnell, Applied microbiology, Giessen University) were used to amplify 16S rRNA

gene fragments (~366bp). PCRs was performed in a 50 µl mixture containing 1.0 µl of each forward and reverse primers (10 µmol·L^{-1}), 1.0 µl template DNA, 2.0 µl DMSO, and 20.0 µl *Premix Taq* DNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian). Cycling conditions included: an initial denaturation at 94℃ for 5 min; 15 cycles of a denaturation step at 94℃ for 30 s and a Touch-down PCR; an extension step at 72℃ for 40 s. Touch-down PCR conditions were used for the initial annealing temperature of 65 for 30 s with a decreasing temperature step of 0.5℃ every two cycles until the final annealing temperature reached 60℃. Amplified fragments were purified with Universal DNA Purification Kit (TIANGEN Biotech Co. Ltd., Beijing) and ligated into pGEM-T easy vector (Promega, Beijing). The ligation products were transformed into *Escherichia* coli JM109 competent cells. Transformants were spread on LB agar containing ampicillin (100 µg ml^{-1}), 40 µl of 20 mg ml^{-1} of X-Gal and 160 µl of 200 mg ml^{-1} of IPTG, then about 200 positive white clones were randomly picked and rRNA gene inserts were confirmed using PCR with universal primers M13F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M13R (5'-GAGCGGATAACAATTTCACACA GG-3'). Thus six 16s rRNA gene libraries corresponding to each slurry were constructed.

RFLP and diversity analysis

The FAc12-66F and FAc12-432R – screened positive colonies PCR products were digested with restriction endonucleases *Mn*l I and *Msp* I at 37℃ for 6 h. Digested RFLP fragments were sepa rated on a 12% polyacrylamide gel running in 1×TBE buffer at 120 V for approximately 2 h. Electrophoresis map was available after silver staining. Fragments shorter than 50 bp were not taken into consideration because of their being close to the detection threshold. Clones with the same RFLP patterns were regarded as one OTU. Library clone number and OUT number were imported into an online tool to evaluate the library size by coverage and predicted numbers of phylotypes (Kemp and Aller, 2004, http://www.aslo.org/lomethods/free/2004/0114a.html). In addition, Alfa-diversity indices including Shannon's index (*H*') (Spellerberg and Fedor, 2003), Simpson's index (*D*s) (Hunter and Gaston, 1988) and Margalef index (D_{Ma}) (Margalef, 1958; Magurran, 2004) were calculated to reflect the community diversity of *Anaeromyxobacter* spp. We also evaluated the niche breadth of the major *Anaeromyxobacter* spp. types that give indications of the number of resources they exploited and their environmental frequencies (Ludwig and Reynolds, 1988) by using the equations developed by Levins (1968) and Colwell (1971).

Sequencing and phylogenetic analysis

One or two representative clones of dominant OTUs were sequenced (GenScript Biological Technology Co., Ltd., Nanjing) and the presence of possible chimeric sequences was investigated by using the CHIMERA_CHECK program of the Ribosomal Database Project (RDP) II. Through NCBI BLASTN searching, closest relatives to our clone sequences were extracted. The obtained sequences were manually proofread and corrected if necessary, edited, and aligned using All sequences were aligned by ClustalX (version 1.83) and phylogenetic tree was constructed by Evolutionary Genetics Analysis (MEGA4.0) software using neighbor-joining method. Robustness of derived groupings was tested by bootstraping 1,000 times. The Newick file of phylogenetic tree was submitted to the Unfrac site (http://128.138.212.43/cgi-bin/fastunifrac/fastunifrac_search.py/handle_prepro?action=display, (Lozupone and Knight, 2005; Lozupone et al., 2006) for Unifrac significance computation and Jackknife Environment Clustering.

Abundance detection

Real-time PCR was employed to measure the 16S rRNA gene copies of *Anaeromyxobacter* spp. and total *Bacteria* with primers FAc12-66F (5'-CGA GAA AGC CCG CAA GGG-3') / FAc12-432R (5'-CGT CCC TCG CGA CAG TGC-3') (Treude et al., 2003) and COM1 (5'- CAG CAG CCG CGG TAA TAC -3') / COM2(5'- CCG TCA ATT CCT TTG AGT TT -3') (Schwieger and Tebbe, 1998) respectively. For a comprehensive and clear comparison, we applied the abundance of *Anaeromyxobacter* spp. as the proportion of its 16S rRNA gene copys to that of *Bacteria* in corresponding incubation. Reaction mixture (50 µl) contained 12.5 µl SYBR® *Premix EX Taq*™ II (TaKaRa Biotechnology Co. Ltd., Dalian) 0.5 µl of each forward and reverse primers (10 µmol·L^{-1}), 2.0 µl template DNA, and 25.0 µl steriled, nuclease-free water. Master mixture was prepared prior to the addition of DNA template. The optimal primer conditions were determined experimentally by using the protocol provided with the master mixture. The tube containing the master mix was vortexed to assure a homogeneous solution and briefly spun down in a microcentrifuge. Cycling conditions included: an initial denaturation at 95℃ for 3 min; 40 cycles of a denaturation step at 95℃ for 30 s, annealing at 55℃ for 30 s, and extends at 72℃ for 35 s; an melting step at 75 to 95℃ for 40 s. Calibration curve was prepared as follows: constructing recombinant containing the amplified 16S rRNA gene fragment of *Anaeromyxobacter* spp. and transforming into JM109, extracting the recombined plasmid DNA, determining its concentration, calculating the copy number according to the equation described by Kolb et al. (2005), diluting plasmid DNA in 10-times serial and taking as templates for real-time PCR, determining the copy numbers and generating the standard curve. Reaction mixture without DNA template was taken as negative control. All tubes were maintained on ice and in the dark during transport to the spectrofluorimetric thermal cycler (Bio-Rad CFX96™, Bio-Rad). Significance tests of Data sets were dealt with the Statistical Package for Social Science (SPSS 13, SPSS Inc., Chicago, IL, USA) with default settings.

Canonical correspondence analysis

Canonical correspondence analysis (CCA) is the most widely used multivariate technique for constrained ordination (Zhu et al., 2005). In our study, primary analysis showed some relations between the samples and the environmental factors were nonlinear. A CCA using CANOCO 4.5 with Monte Carlo permutation test based on 499 random permutations (Ter Braak and Šmilauer, 2002) was therefore used to summarize the data set and to evaluate the expected relationships between the clustering of samples and their environmental factors including Fe (II) concentration, *Anaeromyxobacter* abundance and Shannon's diversity index. Species data was square root-transformed. The results of the analyses were visualized in the form of biplot using the Canodraw for Windows program (Ter Braak and Šmilauer, 2002).

RESULTS

Ferrous iron accumulation

Along with flooding time, the concentration of Fe (II) obviously increased from 1 h to 8 days and reached the plateau after 15 days (Figure 1). The dynamics of ferrous iron in treatments were well fitted by logistic models, which showed that the maximum Fe (III) reduction potential (V_{max}) was 8.27 mg·g^{-1} and the corresponding time of the V_{max} (T_{Vmax}) was 3.36 days. Since Fe(III)

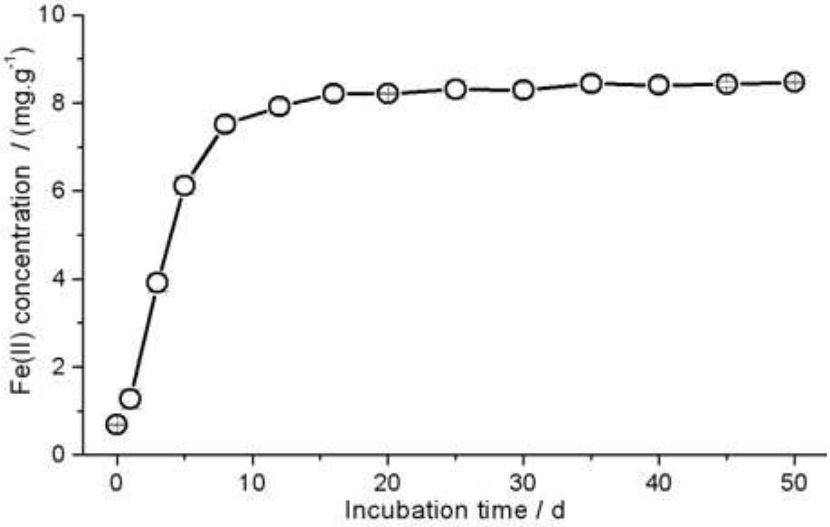

Figure 1. Variation of Fe (II) concentrations in anaerobic paddy soil slurry over flooding time.

Table 1. α diversity indices of *Anaeromyxobacter* spp. 16S rDNA gene in six flooded paddy slurries.

Treatment	OTU number/total clones	Coverage value (%)	Diversity index		
			Shannon index (H'.)	Simpson index (D_s)	Margalef richness index D_{ma})
1 h	43/173	89.60	3.0517	0.9174	8.3442
1 days	42/165	86.06	2.9570	0.8978	8.2257
5 days	43/198	86.87	2.8358	0.8852	8.1312
10 days	49/165	84.24	3.2213	0.9296	9.5967
20 days	39/164	84.15	2.6692	0.8802	7.6473
30 days	45/133	80.45	3.2448	0.9391	9.2018

reduction in anoxic sedimentary environment is confirmed as an enzymatic process related to the growth and activity of relative Fe(III) reducers (Lovley, 1991; Lovley et al., 2004), the accumulation of Fe(II) here also clearly reflected the growth of some microbes.

RFLP analysis of 16S rDNA library

A total 998 16S rDNAs clones of 6 samples were analyzed by PCR–RFLP with restriction enzymes *Mn*l I and *Msp* I, whereby 261 unique RFLP fingerprints (operational taxanomical unit, OTU) were identified (Table 1). Representative clones of major OTUs were sequenced. Sequences were deposited in Genbank with the following accession numbers: JF897864 - JF897922. Following statistics analysis of RFLP lined the treatments of different flooding time in a decreasing order based on their OTU numbers as: 49 at 10 days, 45 at 30 days, 43 at 1 h, 43 at 5 days, 42 at 1 day, and 39 at 20 days (Table 1). Single OTU, whose band pattern appeared only once, showed different proportion to total OUT in flooding incubation over time (Figure 3). Treatment of 30 days contained the highest proportion of single OTU as 19.55%, followed by 15.85% of 20 days, 15.76% of 10 days, 14.46% of 1 day, and 13.13% of 5 days while the lowest proportion 10.40% was found in sample of 1 h.

Considering all the OTUs information and the convenience for describing, major OTUs were selected as the ones whose individuals to total clones were greater than 5.5% in our study and 10 major *Anaeromyxobacter* types were consequently generated and designated as P1 to P10 (Figure 2), while the proportion and distributions of these major *Anaeromyxobacter* types in each library were uneven and discontinuous. The phylogenetic status of these major OTUs will be described later in the study.

Coverages of 6 rDNA libraries ranged from 80.45 to 89.60% (Table 1), indicating that the clones used from each library's screening could well cover the diversity of 16S rRNA genes of *Anaeromyxobacter* spp. Flooding treatments were ordered decreasingly as 30 days > 10 days > 1 h > 1 day > 5 days > 20 days according to their diversity indices of Shannon (*H'.*) and Simpson (*D*s)

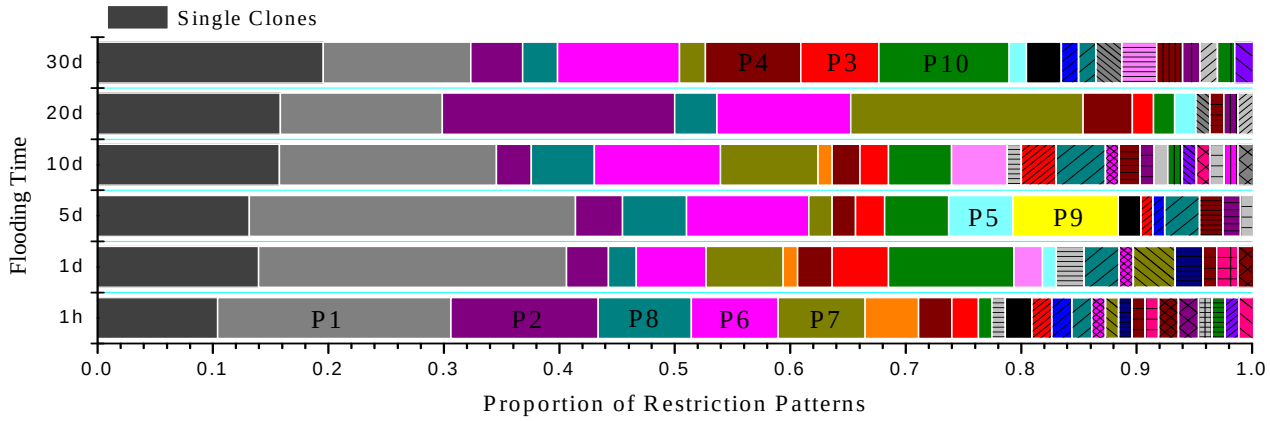

Figure 2. The OTUs frequency of *Anaeromyxobacter* 16S rRNA gene in paddy soil slurry of different flooding time.

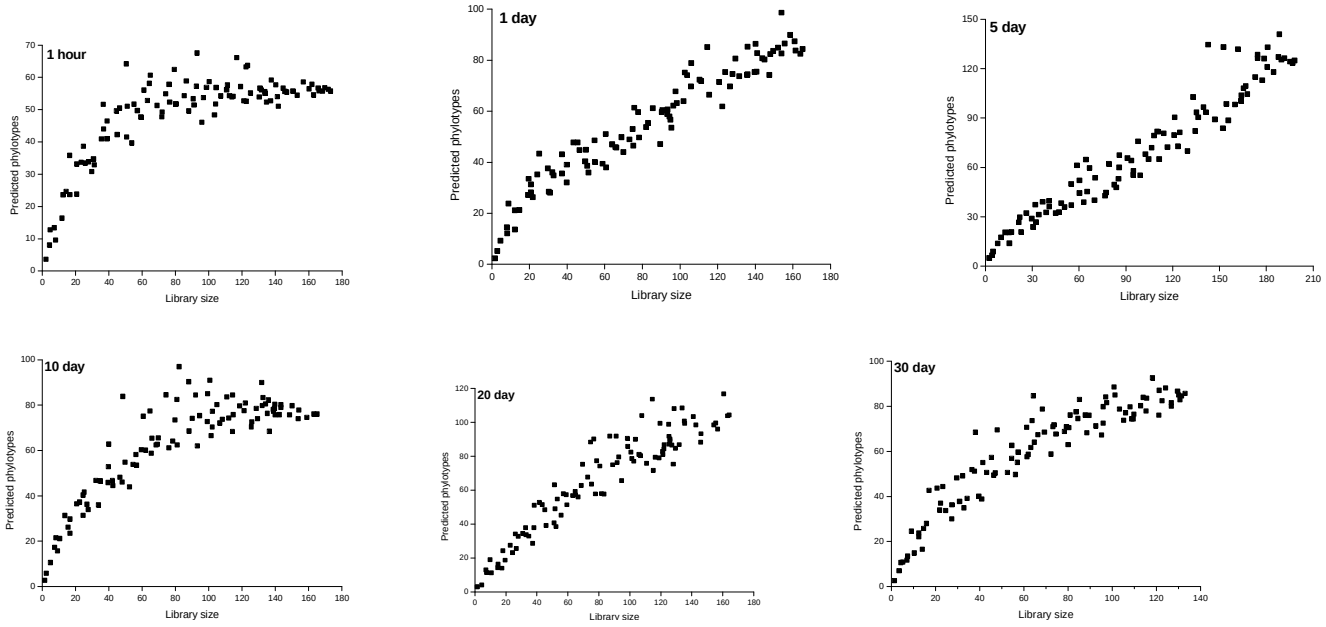

Figure 3. Predicted number of phylotypes versus size of subsamples of six libraries of different flooding time. Each point is the mean of 10 replicate subsamples of the library.

while the order would be 10 days > 30 days > 1 day > 1 h > 5 days > 20 days based on Margalef richness index (D_{Ma}) (Table 1). Phylotypes number prediction (Figure 3) showed 16s rDNA libraries could also reflect the difference on the maximal potential *Anaeromyxobacter* community diversity caused by flooding time, in which the *Anaeromyxobacter* species in slurries of 1, 5 and 20 days were more active on the emerging of new types.

Previous studies (Cole et al., 1994; He and Sanford, 2003; Treude et al., 2003) have demonstrated that *Anaeromyxobacter* spp. could grow optimally without oxygen at all. He and Sanford (2003) also found that as a strategy for *A. dehalogenans* 2CP-C to access crystal Fe (III) oxides, anthraquinone 2, 6-disulfonate (AQDS), as a humic acid analog, could be utilized as electron shuttle, which suggested that humic acid could enhance the reduction of crystal Fe (III) oxides. Generally, crystal Fe (III) oxides are comparatively low in bioavailability, thus will be left as potential resource of Fe (III) reduction in slurry at the late flooding stage. Thirty days flooding could assure the complete depletion of the oxygen and organic material, leave comparatively stable content of humic acid in slurry and therefore motivated the growth and division of the *Anaeromyxobacter* community. The

Table 2. *Unifrac significance*[a] (*P*) of *Anaeromyxobacter* communities in slurries of different flooding time with number of permutations set to 100.

Treatment	10 days	1 days	1 h	20 days	30 days	5 days
10 days	0	0.92	0.14	0.71	0.52	0.67
1 days	0.92	0	0.44	0.64	0.36	0.08
1 h	0.14	0.44	0	0.99	0.12	0.01
20 days	0.71	0.64	0.99	0	0.1	0.15
30 days	0.52	0.36	0.12	0.1	0	0.2
5 days	0.67	0.08	0.01	0.15	0.2	0

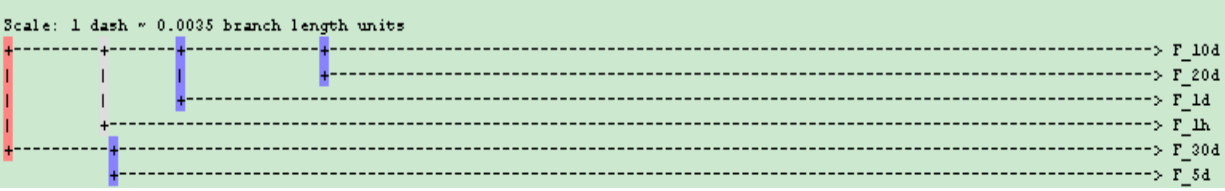

Figure 4. Result of running Jackknife environment clusters based on the phylogenetic information in slurries of different flooding time. The environment abbreviations are the flooding incubations of specific time points where the sequences were retrieved, in which the "F" means flooding, "h" and "d" represents "hour" and "day" respectively. Each node is colored by the fraction of times it was recovered in the jackknife replicates. Nodes recovered >99.9% of the time are red, 90–99.9% are yellow, 70–90% are green, 50–70% are blue, and < 50% are grey.

diversity and richness of *Anaeromyxobacter* community decreased in slurry incubation from 1 h to 5 days (Table 1). However, this slight change indicated an enrichment of some specific members of *Anaeromyxobacter* spp. leaning to take amorphous ferric as electron acceptor and being facultative anaerobic when there are still amorphous ferric and partial soluble oxygen in the soil at initial flooding stage. The fluctuation of diversity observed in later flooding stage require for further study.

Phylogenetic comparison of *Anaeromyxobacter* community

UniFrac analysis, based on phylogenetic information, was performed to directly compare how similar or dissimiliar *Anaeromyxobacter* communities were in slurries of different flooding time. By testing each pair of flooding incubations, no highly significant difference was found (Table 2). Only the flooding incubations of 1 h and 5 days showed significant (*P* = 0.01) difference between their *Anaeromyxobacter* community compositions. There were suggestive differences (*P* = 0.09) between the *Anaeromyxobacter* communities of 1 and 5 days flooding incubations, as well as between incubations of 20 and 30 days old (*P* = 0.1). This supposed to be prospective, considering that we observed the *Anaeromyxobacter* on genus level using specific primers and thus avoided the possible huge variations when analyzing the whole *Bacteria* (Noll, 2005; Valenzuela-Encinas et al., 2009). However, the marginally significant difference (between samples of 1h and 5 days) and suggestive differences (between 1 h and 30, 5 and 20 days) of *Anaeromyxobacter* community structures, on some extent, suggested the existence of community regulation by *Anaeromyxobacter* itself adapted to the environmental variation over flooding periods.

Using *Jackknife Environment Clustering* in UniFac, we cluster the slurries of different flooding time (Figure 5). The clustering suggested that the flooding time had certain control on the Phylogenetic constitution in slurries. Sequences from the slurries of 1 h, 1, 10 and 20 days old were more similar to each other than they were to sequences from slurries at 5 and 30 days, since they clustered together to the exclusion of the 5 and 30 days slurries and was highly supported (> 99.9% of the time). Slurries at 10 and 20 days fell together as a group, as well as incubations of 5 and 30 days, both of which were supported > 50% of the time. Slurry at 1 day was close to the group contained slurries of 10 and 20 days, while 1 h slurry was excluded from the former three samples, whereas the lack of bootstrap support (< 50%) indicated that more sampling would be required for confidence in this result. Apparently, phylogenetic compositions in slurries could be gathered according to the flooding periods they belonged to (Figure 4). This observation shared some common trait with Noll's (2005) work that the shift of microbial community was in different manner at different flooding stage. Three phases were given in Noll's (2005) report, including phase I (1 h to 2 days), Phase II (2 to 21 days) and Phase III (21 to 168 days), while three clusters were also displayed in our study.

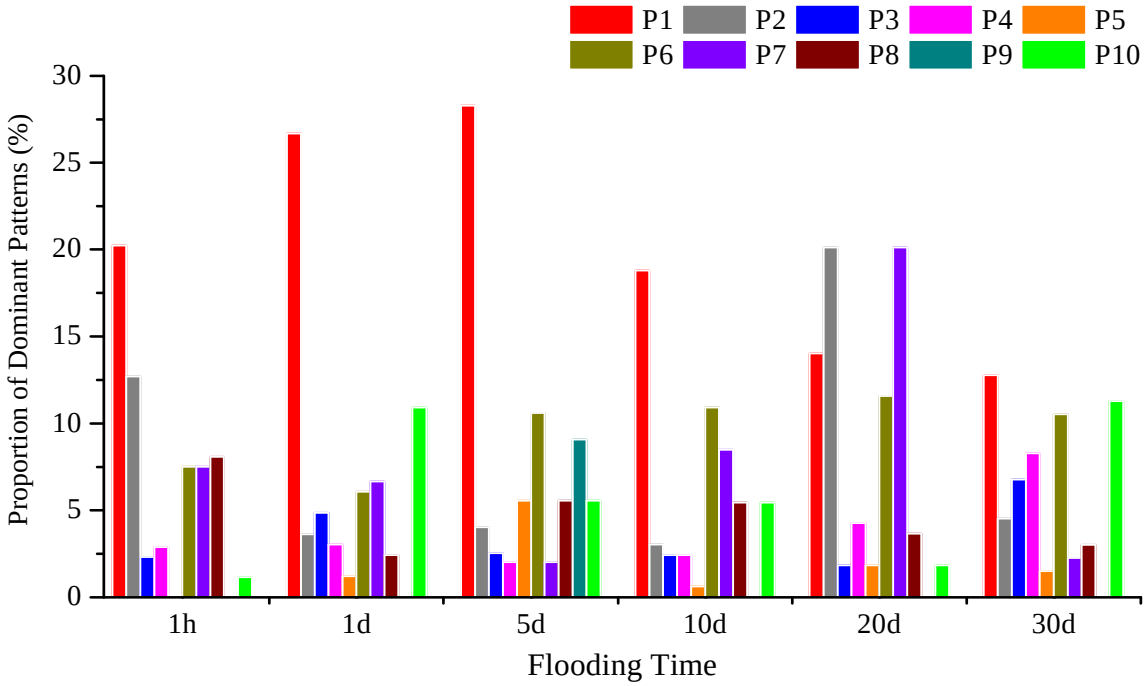

Figure 5. Successional dynamics of major types of *Anaeromyxobacter* in paddy soil slurries over flooding time.

This might just be coincidence, but allow us to assume that the environmental conditions, changing with the increasing flooding time, selected *Anaeromyxobacter* spp. by their adaptability and thus decided their community structure. Furthermore, our result of CCA supported this assumption, confirming that the environmental factors like Fe (II) content and *Anaeromyxobacter* abundance and its diversity influenced the grouping of slurry based on flooding time (Figure 7).

Successional dynamics of major *Anaeromyxobacter* communities and their niche breadth analysis

P1(red bar) was apparently visualized as increasing from 1 h to 5 days and decreasing in the rest time, while it was the biggest *Anaeromyxobacter* type in all slurries except for the one at 20 days (14.02%). P6, as the second stable type, kept its population size in all slurries averagely as 9.54% and showed an increase from 1 day (6.06%) to 20 days (11.59%) and then slight fell to 10.53% at 30 days in spite of its subtle declining from 1 h to 1 day. Type P7 and P10 were quite different in growth trend, as the former decreased from 1 h (7.51%) to 5 days (2.02%) and increased from 5 days to 20 days (20.12%) then again fell to 2.26% at 30 days, while the latter was firstly increased by almost 9 times to 10.91% as the second higher type at 1 day and kept on falling to 1.83% at 20 days then increased again to 11.28% at 30 days. It seemed there was kind of competitive relationship between them. As the second higher *Anaeromyxobacter* type (12.72%) at 1 h, P2 was then staying low as 3.57% on average during 1 day to 10 days and was the highest (20.12%) at 20 days like P7, but downsized to 4.51% at 30 days. Both P3 and P4 existed at a comparative low level around 2.79 and 2.93% on average during 1 h to 20 days and grew to 6.77 and 8.27% at 30 days, respectively. The highest population size of P8 was observed at 1 h as 8.09% and lived as minor type in the rest days. P5, as the type undetected at 1 hour, was only relatively higher at 5 days (5.56%) and quite low at other times (1.29% on average). Strikingly, P9 was only found and ranked the third in population size (9.09%) in slurry at 5 days.

The diversity data (Table 1) and data of 16S rDNA copy number (Table 4) suggested that the principles of r- and K-selection (Andrews et al., 1986) can be applied to the succesional dynamics of major *Anaeromyxobacter* types in slurry. For instance, The highest diversity value and the comparatively lower 16S r RNA gene copy numbers ($1.748 \times 10^5 \pm 1.050 \times 10^5$ copies·g^{-1} dried soil) at 30 days (Table 4) enabled us to refer that the *Anaeromyxobacter* species preferred to keep population by maintaining species rather than by ensuring individual numbers in this stage. This assumption fitted well to describe K- selection (Andrews et al., 1986) microbes. In the contrast, the diversity value of *Anaeromyxobacter* spp. in 20 - days slurry was the lowest (H'. = 2.6692; D_s = 0.8802; D_{ma} = 7.6473) while its absolute 16S r RNA gene copy numbers was the highest as $6.162 \times 10^5 \pm 1.403 \times$

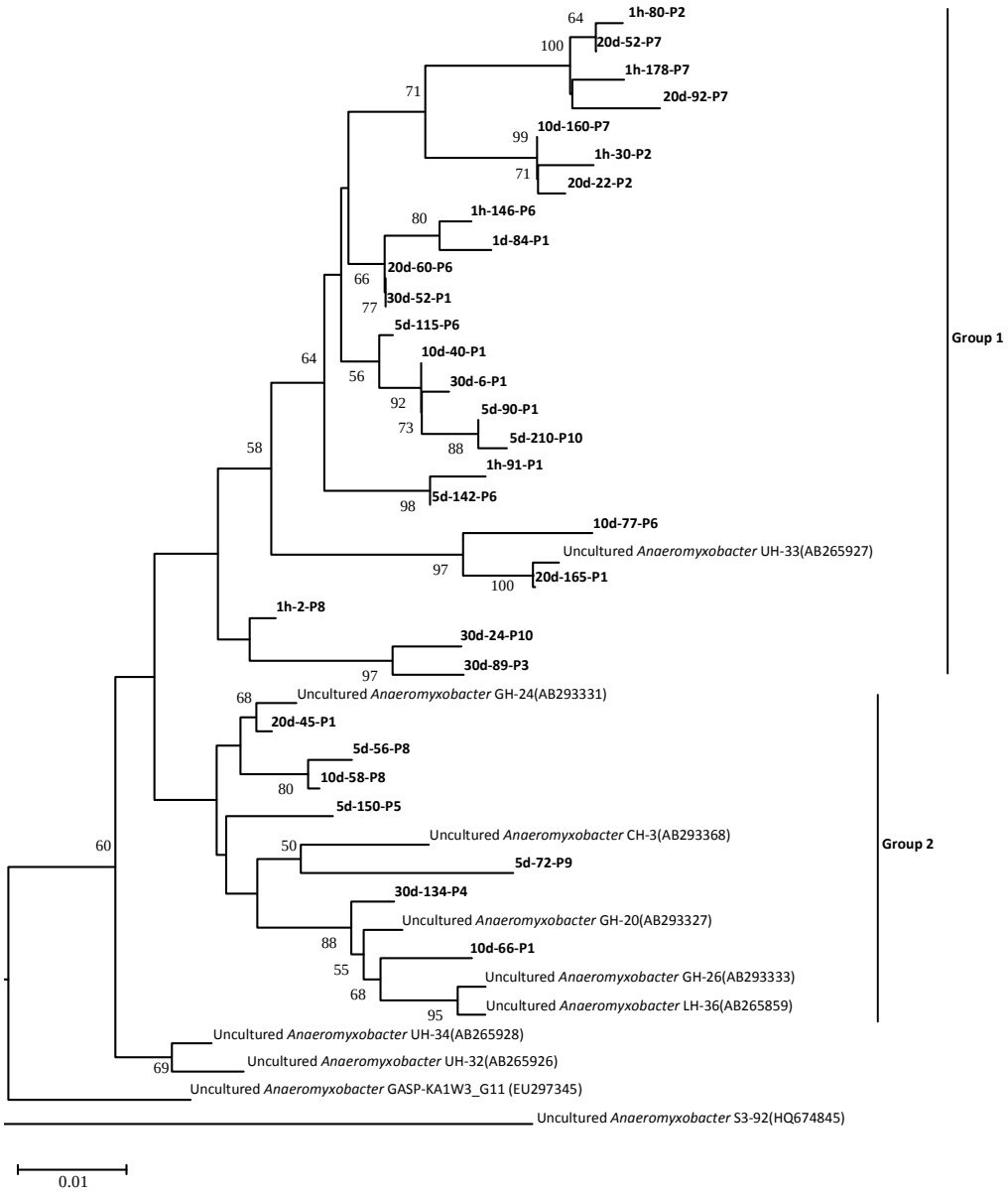

Figure 6. 16S rRNA gene-based phylogenetic tree of environmental clone sequences of major *Anaeromyxobacter* types with Neighbor-Joining method. Bootstrap values of >50% (1000 replications) were shown at the branches, GenBank accession numbers of candidate sequence were in the parentheses, the boldfaces indicated clone sequences obtained in this study. The scale bar represents 0.001 sequence divergence.

10^5 copies·g^{-1} dried soil, which indicated that the *Anaeromyxobacter* spp. in this condition inclined to proliferate more individual within a less species, as the performance of r- selection microbes. At the initial flooding stage (1 h to 5 days), a comparative plenty of carbon resource and ferric oxides facilitated the fast increase of *Anaeromyxobacter* spp. adopting r-selection and thus resulted in the appearance of major types like P1 and P6. During the late stage of flooding (10 to 30 days), with the consumption of acetate to its plateau (Peng et al., 2008), the competition for electron donors aggravated, and then species of K-selection would replace the species of r-selection subsequently. As observed, being possible K-selection models, P3 and P4 increased their proportions in slurry at 30 days after staying on a low level, while the proportion of P1 and P6 decreased a little.

However, resource exploitation capabilities of major

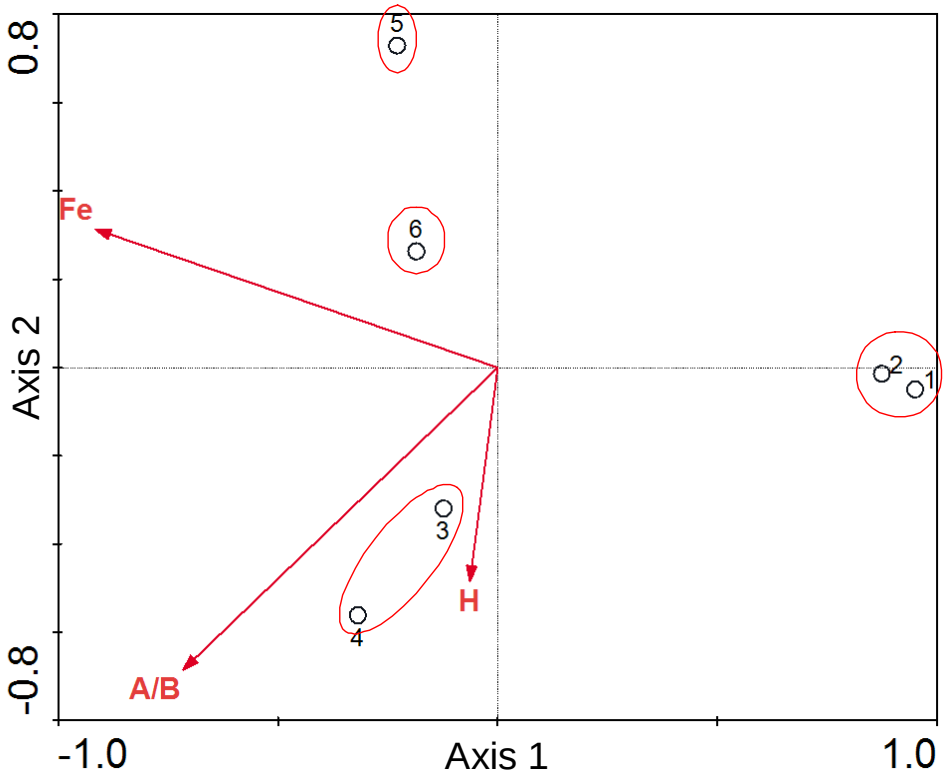

Figure 7. CCA Biplot based on a canonical correspondence analysis of the slurries of different flooding time with respect to the environmental factors (arrows). (Fe, the content of Fe (II); H, Shannon's index; A/B, *Anaeromyxobacteria* abundance). The magnitude of variation between the environmental factors is depicted in a two-dimensional space. The plot represents 100% of the original variation in the data; axis 1 and 2 explain 85.5 and 13.9 % of this variation, respectively. Empty circles 1 to 6 represent the slurries of 1 h, 1, 5, 10, 20 and 30 days old. Monte Carlo permutation test on significance of all canonical axes showed P = 0.002.

Anaeromyxobacter types adjusted to environmental availability during flooding incubation were measured by Levins' and Shannon-Wiener's indices of niche breadth and identical results were showed (Table 4). In major *Anaeromyxobacter* types, P6 had the highest niche breadth value while P1, P3 and P4 were relatively higher (*BL* = 5.041 ~ 5.255; *BS* = 1.707 to 1.720). P9 possessed the lowest niche breadth (*BL* = 1; *BS* = 0) and P5 (*BL* = 1.352; *BS* = 0.429) was slightly higher than P9. Niche breadth or niche width is a concept underlying several hypotheses in evolutionary ecology (Feinsinger et al., 1981). Abiotic and biotic factors are considered to modify niche breadth in ecological and evolutionary terms (Feinsinger et al., 1981), and the evaluation of niche breadth depends on the variety of resources available and their selection by the consumer (Petraitis, 1979). Thus, niche breadth quantifies the degree of specialization presented by a species relative to resource availability. So P6, P1, P3 and P4, which might use a wide variety of resources, were more competitive than others in adapting to the environment variation and expanding their existence to the whole flooding period with more individuals. P9 and P5, in our observation, behaved as specialists that appeared only in one or few certain flooding period. In addition, phylogenetic identification showed that type P1 was closely related to sequences that covered from soils amended with goethite or acetate (Figure 7) (Hori et al., 2010). This also explained why P1 was so widely and abundantly distributed during the whole flooding incubation and supported the niche breadth analysis. Major type P9, the one only appeared in slurry at 5 days was closely related to sequence retrieved from paddy soil of Italy (Hori et al., 2010). This suggested the existence of specific *Anaeromyxobacter* spp. in paddy soils from different area and the comparison of *Anaeromyxobacter* communities in various paddy soils would benefit us on isolating new strains.

Phylogenetic analysis of major *Anaeromyxobacter* types

One or two clones of major *Anaeromyxobacter* types (P1

Table 3. Niche breadth of major *Anaeromyxobacter* types during flooding incubation.

Niche breadth index (*B*)	Number of major type									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Levins (*BL*)	5.255	3.691	5.161	5.041	1.352	5.673	3.803	4.944	1	4.403
Shannon-Wiener (*BS*)	1.720	1.512	1.713	1.707	0.429	1.762	1.525	1.685	0	1.587

Table 4. 16S rDNA copies of *Anaeromyxobacter* and *Bacteria* in slurries of different flooding time.

Flooding treatment	16S rDNA copies·g^{-1}dried soil		*Anaeromyxobacter* Abundance (*A*/*B*) (%)
	Anaeromyxobacter (×10^5)	*Bacteria* (×10^8)	
1 h	1.150±0.710 ^{d}	4.744±0.157 ^{a}	0.024
1 days	1.743±0.508 ^{c}	6.917±2.220 ^{a}	0.025
5 days	2.137±0.505 ^{b}	0.560±0.039 ^{b}	0.356
10 days	2.159±0.399 ^{b}	0.420±0.069 ^{b}	0.514
20 days	6.162±1.403 ^{a}	5.292±0.110 ^{a}	0.116
30 days	1.748±1.050 ^{c}	0.615±0.047 ^{b}	0.284

to P10) derived from RFLP profiling were chosen for sequencing and evolutionary distance analysis (Figure 6). Phylogenetic tree showed that major *Anaeromyxobacter* types detected in this study were affiliated with the clone sequences from anoxic flooding paddy soils of *Anaeromyxobacter* which was capable of assimilating acetate and dissimilatorily reducing Fe (III) (Hori et al., 2007; Hori et al., 2010). Phylogenetic placement confirmed the specificity of the primer system. Bootstrap values reinforced that representatives of two distinct *Anaeromyxobacter* clusters, designated Group1 and Group2. These clones were related to isolated strain *Anaeromyxobacter* FAc12 (92 to 98% sequence identity) and *Anaeromyxobacter* 2CP-C (92 to 99% sequence identity). Major types P1 and P8 were distributed in both Group 1 and group 2, whereas P1 was preferential to locate in Group1. As the larger cluster that gathered basically only the clones recovered from our study, Group 1 exclusively contained dominant patterns P2, P3, P6, P7, and P10 while P1, P6, and P7 formed their own tight clusters. Group 2 was the collection of P4, P5, P8 and P9 and most of the candidate sequences selected from NCBI (National Center for Biotechnology Information). In addition, Group 2 was on some extent distinct from Group 1 by only harboring sequence clones from 16S rDNA libraries of slurries at 5 and 30 days, while Group1 contained sequences of environmental *Anaeromyxobacter* spp. from all the slurries. Group1 was almost the collection of the environmental clone sequences retrieved from our experiments, besides one candidate sequence from NCBI. Similar results were also found when analyzed the evolutionary relationships among the uncultured environmental clones (Hori et al., 2010; Thomas et al., 2009; Treude et al., 2003), this niche-based convergent suggested the significant influence of environment on the formation of unique cluster.

Abundance variation of *Anaeromyxobacter* spp. in slurry over time

As shown in Table 3, the number of *Anaeromyxobacter* spp. 16S rRNA gene copies per g of dry soil ranged from $1.150 \times 10^5 \pm 0.710 \times 10^5$ to $6.162 \times 10^5 \pm 1.403 \times 10^5$, which showed a significant increase from 1 hour to 20 days and a significant drop from 20 days to 30 days. Meanwhile, the number of *Bacteria* 16S rRNA gene copies per g of dry soil ranged from $0.420 \times 10^8 \pm 0.157 \times 10^8$ to 6.917×10^8, being highest at 1 day and declined to minimum at 10 days. The abundance of *Anaeromyxobacter* spp. in paddy soil varied over flooding time, starting from the lowest point of 0.242% at 1 h to its summit as 5.14% at 10 days, then decreased to 1.16% at 20 days and increased again to 2.84% at 30 days. Observation highlighted the fluctuation of *Anaeromyxobacter* spp. abundance in paddy soils slurry at late stage. Being higher than the result that ranged from 0.0002 to 2.3% in Thomas's study (2009) on sediment contaminated by uranium, the abundances of *Anaeromyxobacter* spp. in our study were between 0.024 and 0.514% in different flooding treatments, hence indicated that the size of *Anaeromyxobacter* spp. were significantly environment-related. From 1 to 5 days, *A/B* rose sharply from 0.025 to 0.356% and was in well correspondence to the maximal rate of Fe (III) reduction observed on 3.36 days, indicating a possibly positive correlation between *Anaeromyxobacter* spp. and Fe (III) reduction, which was also revealed by CCA in Figure 7.

The value of *A/B* slowly increased to 0.514% from 5 to 10 days as the peak point, while the Fe (III) reducing rate was gradually declining. This might be attributed to the increase of *Anaeromyxobacter* spp. that was leaning to use crystalline iron oxides and hence slow down the rate. From 20 to 30 days, 16S rDNA copies of bacteria declined by almost 8 times, as consequence of the depletion of nutrient resource. But *Anaeromyxobacter* abundance increased to 0.284% in this period because of its capability of reducing crystalline iron oxides. This was also found in Hori's study, where *Anaeromyxobacter* occupied 33% in the clone library from paddy field feed with goethite and acetate, being much higher than its proportion (0.05%) in the treatment modified with ferrihydrite and acetate, suggesting that *Anaeromyxobacter* was well capable in reducing crystalline iron oxides. Or say, *Anaeromyxobacter* was disabling to compete for easy using iron oxides with other microbe like *Geobacter.* Although in our study the proportion of *Anaeromyxobacter* spp. to the whole bacteria was too low to explain its contribution to the Fe (III) reduction, at least this genus sustained its abundance by possible reduction of crystalline iron oxides.

Canonical correspondence analysis of environmental factors and slurry clustering

The results of the CCA are shown in Figure 7, where the empty circles tagged with number 1 to 6 on the graph represent paddy soil slurries sampled at different flooding time. Arrows representing *Anaeromyxobacteria* abundance and Fe (II) content had the longest lines and therefore exhibited their strongest correlations with the distribution of slurries of different time in plot, namely these two factors clearly influenced the pattern of the paddy soil slurry during incubation. The angles between the lines show how much the attributes of environmental factors are interrelated; the closer the angles, the greater the interrelation. For instance, the *Anaeromyxobacteria* spp. abundance had a stronger interrelation with Shannon's index than with Fe (II) content, while basically no correlation was found between Fe (II) content and Shannon's index. Slurries at different time distributed in the ordination plot as four distinct groups as slurries of 1 h and 1 day old, slurries at 5 and 10 days, slurry at 20 days and slurry at 30 days. Flooding time thus appeared to change the Fe (II) content, the abundance and diversity of *Anaeromyxobacteria* spp. and thus controlled the clustering of slurries.

While the reasons and mechanisms for the observed successional dynamics and heterogeneous presences of *Anaeromyxobacter* spp. in paddy soil of different flooding periods require further investigation, this study provides valuable information about the growth orbits of the major members and the community diversity information over flooding time, facilitating the isolation of new *Anaeromyxobacter* strains, especially when considering the level of metabolic diversity within this community is unclear due to a limited number of isolated representatives.

ACKNOWLEDGEMENTS

The authors thank Prof. Sylvia Schnell and Dr. Stefan Ratering for their help with literature supporting and primer sequence supply. Our gratitude also goes to Jiaohua You due to his participation in DNA extraction. This work was supported by program of National Natural Science Foundation of China under contract 40971158.

REFERENCES

Andrews JH, Harris RF (1986). r- and K-selection and microbial ecology. Adv. Microb. Ecol., 9: 99-144.

Cole JR, Cascarelli AL, Mohn WW, Tiedje JM (1994). Isolation and characterization of a novel bacterium growing via reductive dechlorination of 2-chlorophenol. Appl. Environ. Microbiol., 60: 3536-3542.

Colwell RK, Futuyma DJ (1971). On the measurement of niche breadth and overlap. Ecology, 52: 567 - 576.

Feinsinger P, Spears EE, Poole RW (1981). A simple measure of niche metrics. Ecology, 62: 27 - 32.

Gelsomino A, Badalucco L, Ambrosoli R, Crecchio C, Puglisi E, Meli SM (2006). Changes in chemical and biological soil properties as induced by anthropogenic disturbance: A case study of an agricultural soil under recurrent flooding by wastewaters. Soil Biol. Biochem., 38: 2069 - 2080.

Haddad A, Camacho F, Durand P (1995). Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete Alvinella pompejana. J. Appl. Environ. Microbiol., 61: 1679-1687.

He JZ, Qu D (2008). Dissimilatory Fe(III) reduction characteristics of paddy soil extract cultures treated with glucose or fatty acids. J. Environ. Sci., 20: 1001 - 0742.

He Q, Sanford RA (2003). Characterization of Fe (III) reduction by chlororespiring *Anaeromyxobacter dehalogenans*. Appl. Environ. Microbiol., 69: 2712-2718.

He Q, Sanford RA (2002). Induction characteristics of reductive dehalogenation in the *ortho*-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans.* Biodegradation, 13: 307-316.

He Q, Yao K (2010). Microbial reduction of selenium oxyanions by *Anaeromyxobacter dehalogenans*. Bioresour. Technol., 101: 3760-3764.

Hori T, Müller A, Igarashi Y, Conrad R, Friedrich MW (2010). Identification of iron-reducing microorganisms in anoxic rice paddy soil by ^{13}C-acetate probing. FEMS Microbiol. Ecol., 4: 267 - 278.

Hori T, Noll M, Igarashi Y, Friedrich MW, Conrad R (2007). Identification of acetate-assimilating microorganisms under methanogenic conditions in anoxic rice field soil by comparative stable isotope probing of RNA. Appl. Environ. Microbiol., 73: 101-109.

Hunter PR, Gaston MA (1988). Numerical index of the discriminatory ability of typing systems-an application of Simpson's index of diversity. J. Clin. Microbiol., 26: 2465 - 2466.

Kemp PF, Aller JY (2004). Estimating prokaryotic diversity: When are 16S rDNA libraries large enough? Limno. Oceanogr-Meth., 2: 114 - 125.

Kolb S, Carbrera A, Kammann C, Kämpfer P, Conrad R, Jäckel U (2005). Quantitative impact of CO_2 enriched atmosphere on abundances of methanotrophic bacteria in a meadow soil. Biol. Fertil. Soils, 41: 337-342.

Levins R (1968). Evolution in changing environments. Princeton University Press, Princeton. New Jersey, USA.

Lin B, Christelle H, Steeve B, Martin B, Philippe VC, Wilfred FMR (2007). Phylogenetic and physiological diversity of dissimilatory ferric iron reducers in sediments of the polluted Scheldt estuary, Northwest Europe. Environ. Microbiol., 9: 1956-1968.
Lovley DR (1991). Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Rev., 55: 259-287.
Lovley DR, Holmes DE, Nevin KP (2004). Dissimilatory Fe(III) and Mn(IV) reduction. Adv. Microb. Physiol., 49: 219-286.
Lozupone C, Hamady M, Knight R (2006). UniFrac - An online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinf., 7: 371.
Lozupone C, Knight R (2005). UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol., 71: 8228 - 8235.
Ludwig JA, Reynolds JF (1988). Statistical Ecology: A Primer on Methods and Computing. John Wiley & Sons, New York.
Lüdemann H, Arth I, Liesack W (2000). Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. Appl. Environ. Microbiol., 66: 754 - 762.
Magurran AE (2004). Measuring Biological Diversity. Blackwell Publishing, London.
Margalef R (1958). Information theory in ecology. Gen. Syst., 3: 36-71.
Marshall MJ, Dohnalkova AC, Kennedy DW, Plymale AE, Thomas SH, Löffler FE, Sanford, RA, Zachara JM, Fredrickson JK, Beliaev AS (2009). Electron donor-dependent radionuclide reduction and nanoparticle formation by *Anaeromyxobacter dehalogenans* strain 2CP-C. Environ. Microbiol., 11: 534 - 543.
Michael S, Balague V, Pedros-Alio C (2003). Seasonal changes in the taxonomic composition of bacterioplankton in acoastal oligotrophic system. J. Aquat. Microbial. Ecol., 31: 163-174.
Noll M, Matthies D, Frenzel P, Derakshani M, Liesack W (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environ. Microbiol., 7: 382 - 395.
Oliver N, Haag H, Häfner B (2005). A mutation in the 65, 000 Dalton heat shock protein gene, commonly used for molecular identification of non-tuberculous mycobacteria, leads to the misidentification of Mycobacterium malmoenseas *Mycobacterium marinum*. J. Mol. Cell. Probes., 19: 275 - 277.
Peng JJ, Lü Z, Rui JP, Lu YH (2008). Dynamics of the methanogenic archaeal community during plant residue decomposition in an anoxic rice field soil. Appl. Environ. Microbiol., 74, 2894 - 2901.
Petraitis PS (1979). Likelihood measures of niche breadth and overlap. Ecology, 60: 703–710.
Reichardt W, Briones A, de Jesus R, Padre, B. (2001). Microbial population shifts in experimental rice systems. Appl. Soil. Ecol., 17: 151-163.
Sanford RA, Cole JR, Tiedje JM (2002). Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic Myxobacterium. Appl. Environ. Microbiol., 68: 893-900.
Sanford RA, Wu QZ, Sung Y, Thomas SH, Amos BK, Prince EK, Löffler FE (2007). Hexavalent uranium supports growth of *Anaeromyxobacter dehalogenans* and *Geobacter* spp. with lower than predicted biomass yields. Environ. Microbiol., 9: 2885 - 2893.
Schwieger F, Tebbe CC (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16s rRNA gene-based microbial community analysis. Appl. Environ. Microbiol., 64: 4870-4876.
Sergio RM, Sebastián MÁ, Alonso M, Maira P (2004). Factors affecting interpretation of restriction fragment length polymorphism (RFLP) patterns from PCR-amplified bacterial 16S rRNA. World J. Microbiol. Biotechnol., 27: 275 - 284.
Sjoling S, Cowan DA (2003). High 16S rDNA bacterial diversity in glacial meltwater lake sediment, Bratina Island, Antarctica. J. Extremophiles, 7: 275 - 282.
Spellerberg IF, Fedor PJ (2003). A tribute to Claude Shannon (1916-2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon-Wiener' Index. Glob. Ecol. Biogeogr., 12: 177-179.
Ter Braak CJF, Šmilauer P (2002). CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (version 4.5). Microcomputer Power, Ithaca, New York.
Thomas SH, Elizabeth PC, Jardine PM, Sanford RA, Löffler FE (2009). Diversity and distribution of *Anaeromyxobacter* strains in a uranium-contaminated subsurface environment with a nonuniform groundwater flow. Appl. Environ. Microbiol., 75: 3679-3687.
Thomas SH, Sanford RA, Amos BK, Leigh MB, Cardenas E, Löffler FE (2010). Unique ecophysiology among U(VI)-reducing bacteria as revealed by evaluation of oxygen metabolism in *Anaeromyxobacter dehalogenans* strain 2CP-C. Appl. Environ. Microbiol., 76: 176-183.
Treude N, Rosencrantz D, Liesack W, Schnell S (2003). Strain FAc12, a dissimilatory iron-reducing member of the *Anaeromyxobacter* subgroup of *Myxococcales*. FEMS Microbiol. Ecol., 44: 261 - 269.
Unger IM, Kennedy AC, Muzika RM (2009). Flooding effects on soil microbial communities. Appl. Soil Ecol., 42: 1 - 8.
Valenzuela-Encinas C, Neria-Gonzalez I, Alcantara-Hernandez RJ, Estrada-Alvarado I, de la_Serna F, Dendooven L, Marsch R (2009). Changes in the bacterial populations of the highly alkaline saline soil of the former lake Texcoco (Mexico) following flooding. Extremophiles, 13: 609- 621.
Wu QZ, Sanford RA, Löffler FE (2006). Uranium(VI) Reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C. Appl. Environ. Microbiol., 72: 3608-3614.
Yao H, Conrad R, Wassmann R, Neue HU (1999). Effect of soil characteristics on sequential reduction and methane production in sixteen rice paddy soils from China, the Philippines, and Italy. Biogeochemistry, 47: 269-295.
Zhou J, Bruns MA, Tiedje JM (1996). DNA recovery from soils of diverse composition. Appl. Environ. Microbiol., 62: 316-322.
Zhu M, Hastie TJ, Walther G (2005). Constrained ordination analysis with flexible response functions. Ecol. Model., 187: 524 - 536.