pH, phosphorus and C: P dominantly control the community structure of bacteria, fungi, archaea and nitrogen-cycling-associated microbes in an arable chernozem

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Abstract
The ecological characterization of soil microbes in northeastern China is unclear, hindering the sustainability evaluation of current agricultural practices. In the present study, we comprehensively monitored the soil properties and the community structure of bacteria, fungi, archaea, (bacterial and archaeal) nitrifiers and denitrifer in a chernozem in a long-term experiment located in this region, in order to identify the major determinant(s) on soil microbial structure. The results showed that correlation of a range of soil properties with community structure of the six important microbial groups in the present study supported the viewpoint that land use practices regulate soil microbial community structure through changing soil properties. Particularly, the observation that soil microbial community structure differentially diverged with pH and nutrient availability (total P concentration and C: P) suggested that comprehensive management should be designed to develop sustainability of current agriculture from the point of view of microbial community structure.

Key Words
Community structure, inorganic and organic management, soil property, bacterial and archaeal nitrifiers, denitrifier, chernozem

Introduction
Anthropogenic management usually influences soil microbial community structure, which would directly change soil biogeochemical processes. Identification of the determinants which manipulate soil microbial community structure, therefore, may help to develop desirable management practices. Large scale surveys showed that bacterial diversity and community structure were correlated with soil edaphic properties, such as soil pH (Fierer and Jackson 2006). Soil community structure is also suggested to be regulated by soil property in a given landscape (Enwall *et al.* 2007). These results indicate that soil properties may serve as determinant by which anthropogenic management influence soil microbial community structure.

In agriculture, long term experiment is considered as an optimal way to evaluate the sustainability of specific practices (Rasmussen *et al.* 1998). A number of studies were conducted to investigate the impacts of such long-term practices on soil microbe community structure. However, the conclusions are usually controversial. One of the explanations is that the practices and environments are too diverse. The alternative is that the common proxy, such as soil property, is not simultaneously monitored or analysed. Additionally, most of the studies only focussed on one group of microbes (bacteria, fungi or one functional group) which may lead to conclusions that are bias against specific practice.

Northeastern China is the major commodity grain base, producing over 1/3 commodity grain for China. The importance of the agriculture in this region is becoming greater with increasing population. Therefore, assessment and development of the current agricultural practices are urgent. However, little is known about the soil microbial ecology in this region. In the present study, we comprehensively monitor the soil properties and the community structure of bacteria, fungi, archaea, (bacterial and archaeal) nitrifier and denitrifer in a long-term experimental station located in this region in order to identify the major determinant(s) influencing soil microbial structure and provide references for designing more sustainable agricultural practices.

Methods
Experimental setup and sampling
Soils were collected from the field experiment in Key Observation Station of the Harbin Black Soil Ecology, Ministry of Agriculture (45°40'N, 126°35'E). The field experiment was established on a chernozem in 1980, designed to evaluate the sustainability of single and combination fertilization of mineral N, P, K or organic

manure. Each plot is 168 m^2 (5.6 × 30 m). The cropping sequence is wheat-soybean-maize rotation. Mineral fertilizers were applied at the rate of 150 kg N/hm^2 (as urea and ammonium hydrogen phosphate), 75 kg P/hm^2 (calcium super phosphate and ammonium hydrogen phosphate) and 75 kg K/hm^2 (potassium sulfate) for wheat and maize, and 75 kg N/hm^2, 75 kg P/hm^2 and 75 kg K/hm^2 for soybean. Organic manure (as horse manure and at the rate of equal to 75 kg N/hm^2 (ca. 18 000 kg/hm^2)) was applied once before maize sowing in each cropping sequence. In this study, we sampled 4 treatments (i.e. plots with neither organic nor inorganic fertilizer (CK), plots with mineral fertilizers (NPK), plots with horse manure (OM) and plots with both mineral fertilizers and horse manures (MNPK)) which represent low input, mineral, organic and conventional managements on August 1st 2008 when soybean was in grain filling stage. Each soil sample was a homogenized mixture of 5 soil cores with a depth of 20 cm. Soil samples were stored at -80°C for DNA extraction.

Soil property measurement, DNA extraction, amplification and DGGE
Soil pH, moisture, organic carbon, total N and total P were analyzed with standard protocols. DNA was extracted from 0.5-g soil samples using the Fast DNA spin kit for soil (Bio 101, Carlsbad, CA) and the FastPrep-24 instrument according to the manufacturer's instructions. Extracts were characterized by electrophoresis on 1% agarose gels. 16S RNA genes of bacteria and archaea, internal transcribed spacer (ITS) of fungi, ammonia monooxygenase genes of bacterial and archaeal nitrifiers and nitrite reductase genes of denitrifiers were amplified with primer sets and PCR conditions according to the references listed in Table 1. All PCRs were prepared with TaKaRa polymerase and buffer as suggested by instruction and performed on PCT-200 DNA thermal cycler (Bio-RAD, USA). All DGGEs were run with BioRad DeCode system and with the reported conditions in references listed in Table 1.

Data analysis
The management effects on soil properties were analysed with one-way analysis of variance (ANOVA) using SAS v8.0 (SAS Institute, Inc., Cary, NC). The DGGE profiles were digitized with Quantity One v4.6.2. The normalized band density and soil properties were input as species into Canoco for Windows v4.5.1. After checking gradient length with detrended correspondence analysis (DCA), redundancy analysis (RDA) was performed. The correlations of soil to community structures were tested with Monte Carlo permutation procedure. The significances and explanation proportions of variance in microbial community structure by each environmental factor (soil property) were calculated with a forwards selection step.

Results and discussion
Fertilization practices significantly influenced soil pH, moisture, total N, total C, total P, C:N and C:P (Table 2, $P < 0.05$ in all these cases). Horse manure treatment increased while NPK treatment decreased soil pH compared with low input CK, with MNPK treatment in the intermediate. Likewise, horse manure application helped maintain relatively high soil moisture. In comparison with CK, horse manure and NPK treatments had additively positive effects on soil TN and TC, with MNPK being the highest. The C:N calculated with TC and TN was reduced by MNPK treatment. Soil C: P differed substantially among fertilization practices, being in the order of CK > OM > NPK = MNPK.

The differences in DGGE profiles with different fertilizations were detectable by eyes (Figure 1) and could be separated with RDA (data not shown). Monte Carlo tests showed that community structure of bacteria, fungi, arhaea, bactertial and archaeal nitrifier and denitrifier were correlated with several soil properties (Table 3). Among these soil properties, pH, TP and C: P showed consistently close correlations (i.e. greater correlation efficiency and significance) with all community structure investigated. In addition, community structure of fungi and denitrifier were correlated with TN, TC and soil moisture; Bacteria and bacterial nitrifier were correlated with soil moisture; Archaea was correlated with soil moisture and TN; Archaeal nitrifier was correlated with TN and TC.

Collectively, the soil properties monitored in the present study totally explained 75%, 76%, 88%, 98%, 74%, 97% of the total variance of the community structure of bacteria, fungi, archaea, bacterial and archaeal nitrifer and denitrifier, respectively, and pH, TP and C:P accounted for over 71% of the explanation. Particularly, variances in community structure of bacteria, archaea and bacterial nitrifier were mostly explained by pH (with explanation percentage of 41%, 69% and 61%, respectively) while those of fungi, archaeal nitrifier and denitrifier were mostly explained by TP or C: P (with explanation percentage of 45%, 49% and 65%, respectively).

Table 1. Primer sets used in this study and source references

Target group	*First round PCR primer*	*Second round PCR primer*	*Reference*
Bacterial 16S RNA gene	27f/1492r	357f-GC/518r	Muyzer *et al.* 1993
Archaeal 16S RNA gene	109f/1492r	344f-GC/519r	Yu *et al.* 2008
Fungi	ITS1F/ITS4	ITS1F-GC/ITS2	Anderson *et al.* 2003
Bacterial nitrifier	amoA1F-GC/amoA2R		Rotthauwe *et al*. 1997
Archaeal nitrifier	23f/616r		Tourna *et al.* 2008
Denitrifier	F1aCu/R3Cu-GC		Throbäck *et al.* 2004

Table 2. Effect of fertilization regimes (i.e. neither mineral fertilizer nor organic manure (CK), mineral fertilizer (NPK), organic manure (OM) and both mineral fertilizer and organic manure (MNPK)) on soil pH, moisture, total nitrogen (TN), total carbon (TC), total phosphorus (TP), C: N and C:P ratio. Data is the means of 3 replications. Different letters within each column indicate significant different at the level of $P < 0.05$.

	pH	*Soil moisture (%)*	*TN (g/kg)*	*TC (g/kg)*	*TP (g/kg)*	*C: N*	*C: P*
CK	6.03b	18.91b	1.29c	24.69c	0.31c	19.10a	78.77a
OM	6.14a	20.41a	1.47b	27.95b	0.42b	19.01a	66.35b
NPK	5.30d	18.22b	1.44b	27.65b	0.62a	19.14a	45.14c
MNPK	5.43c	18.51b	1.61a	29.07a	0.66a	18.10b	44.48c

Table 3. Correlations between community structure and soil properties. The correlation efficiency (r) and significance (*P* value) were obtained with Monte Carlo permutation test.

Soil properties	Bacteria		Fungi		Archaea		Bacterial nitrifier		Archaeal nitrifier		Denitrifier	
	R	P	r	P	r	P	r	P	r	P	r	P
pH	0.305	0.002	0.332	0.004	0.605	0.002	0.592	0.002	0.336	0.006	0.625	0.002
C:P	0.265	0.002	0.339	0.002	0.515	0.004	0.467	0.002	0.359	0.002	0.608	0.004
Total P	0.255	0.004	0.334	0.002	0.538	0.006	0.462	0.004	0.363	0.002	0.627	0.002
Soil moisture	0.207	0.028	0.185	0.036	0.396	0.012	0.459	0.002	0.159	0.084	0.317	0.034
Total N	0.138	0.206	0.251	0.004	0.335	0.026	0.220	0.064	0.250	0.022	0.428	0.006
Total C	0.119	0.278	0.226	0.020	0.267	0.066	0.227	0.078	0.243	0.020	0.352	0.020
C: N	0.085	0.464	0.161	0.080	0.232	0.098	0.107	0.358	0.114	0.266	0.272	0.700

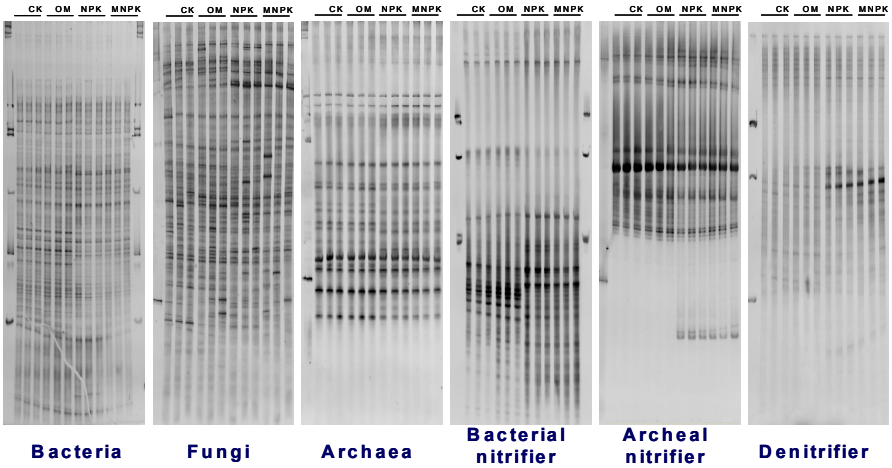

Figure 1. The DGGE profiles of bacteria, fungi, archaea, bacterial and archaeal nitrifiers, denitrifier with neither mineral fertilizer nor organic manure (CK), mineral fertilizer (NPK), organic manure (OM) and both mineral fertilizer and organic manure (MNPK). Each treatment has 3 replications.

As in many other studies which assessed the effects of long term fertilization, our results showed that soil properties with different fertilization regimes changed in a predictable way. For example, nitrogen fertilizer reduced soil pH while organic manure increased soil TC, TN and TP. Previously, such soil properties were mainly related to crop growth and productivity, microbial biomass and activity. In the present study, we

found that soil properties could also explain a large proportion of variance in microbial community structure. pH was recently reported as a strong predictor for DNA-based bacterial community structure in non-agricultural soil at a large scale and across land use types in wetland (Hartman *et al.* 2008). Bacterial community structures in arable soil were also frequently observed to be different in upland arable soil with different pHs caused by fertilizations. Consistently, we found that, in arable chernozem, pH could explain up to 41% of the variation in bacterial community structures in the present soil. By contrast, soil bacterial community structure remained stable after over 160 years of manure and inorganic N amendment if soil pHs were maintained close to neutral (Ogilvie *et al.* 2008). Therefore, the effect of N-mediated pH on bacterial community structure is likely a general consequence in agrosystem.

Additionally, community structure of ammonia oxidizing *Betaproteobacteria*, the key player in nitrogen cycling, is also mainly controlled by pH in the present soil, suggesting that pH not only regulated community structure, but bacterial process in agrosystem. Archaea was recently suggested to take part in nitrification, but the influences of long term fertilization on archaeal community structure were seldomly explored. In grassland, increased pH, application of inorganic fertilizer (ammonium nitrate) and sheep urine did not change overall archaeal community structure significantly. However, subgroup of arhaea, such as crenoarchaea 1.1b and 1.1c seems to be sensitive to pH shift. In the present study, pH was found to be the major regulator of arhaeal community structure. We hypothesize that the components causing changes in archaeal community structure are crenoarchaea 1.1b or 1.1c, or both.

Unlike bacteria, AOB and archaea, community structure of fungi were mostly regulated by soil C: P in the present study. Due to the preference of high phophorus by Ascomycota and low P by Basidiomycota, the community structure of fungi was changed by the variation in P by P fertilizer or land use types (Lauber *et al.* 2008). In addition, because basidiomycota fungal can decompose low quality components in plant litter, fungal community structures is usually shaped by soil C: N (Marschner *et al.* 2003). In our analysis, fungal community structure was not correlated with C: N, which was probably ascribed to a small variation in C:N induced by fertilizations. C: P as the strongest predictor suggested both individual and interacting effect of soil P and carbon concentration shaped fungal community structure under the present condition. Denitrifier community structure was sensitive to a range of factors, such as organic manure addition, plant identity, soil pH, etc. In the current study, we identified that TP was the strongest regulator. Likewise, TP dominated the influences on community structure of archaeal nitrifier which tends to live in habitats of low-nutrient, low-pH, and sulphide containing environments.

Conclusion

Correlation of a range of soil properties with community structure of six microbial groups in the present study supports the view that land use practices regulate soil microbial community structure through changing soil properties. The consistence of the observation that soil microbial community structure diverges with different pHs and nutrient availabilities with literature highlights that comprehensive management should be designed to develop sustainability of current agriculture in the viewpoint of microbial community structure.

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