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Catabolic and genetic diversity of microbial communities in Australian soils are influenced by soil type and stubble management

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Abstract
Crop residues (stubble) are one of the major sources of C in low fertility agricultural soils of Southern Australia. We measured the effect of stubble addition on genetic and catabolic diversity of microbial communities in two soil types, i.e. Alfisol and Vertosol. Catabolic diversity of microbial communities was significantly affected by stubble addition in both soil types. Actinobacteria, Proteobacteria and Acidobacteria accounted for 76% of bacterial populations and bacterial community structure differed between soil types. The effect of stubble addition on the microbial metabolic potential and bacterial community structure was greatest when stubble was incorporated compared to standing stubble. Although stubble management altered microbial diversity, soil type appears to have a dominant influence in determining bacterial community structure in these soils.

Key Words
Bacterial diversity, 16S rRNA, catabolic diversity, *nif*H, soil type, stubble

Introduction

Soil biological function is an outcome of population diversity and activity of microbial communities as limited by edaphic and environmental constraints. In many Australian agricultural soils, carbon availability is the most limiting constraint of microbial function hence management of biologically available carbon is the key to changes in microbial diversity and improvement of function (Gupta *et al.* 2009). Crop residues (stubble) are one of the major C sources in low fertility agricultural soils of Southern Australia. It is generally believed that stubble retention can provide benefits from improvements to different biological properties (Roper and Gupta 1995). However, in different cropping regions of Australia, the nature and extent of these benefits and changes on microbial diversity are unknown. Soil organic C levels in these soils are generally low and soil biota generally experience boom-bust cycles of C availability. The depletion of carbon-rich microsites can affect the distribution, diversity and metabolic status of microbial communities and can impact on the overall biological resilience.

Soil type, residue quality and environment can significantly impact microbial populations and their metabolic activities, potentially influencing the timing and extent of the biological benefits derived from stubble retention. The rate of stubble decomposition during the initial six month period is known to vary with soil type, litter chemistry and climate (temperature and moisture). In a three year study, we monitored changes in soil biological and chemical properties as influenced by stubble management treatments on two soil types. Field based experiments were used to determine stubble effects on soil biological function and to identify linkages with crop yield. Glasshouse experiments were conducted to examine the short-term effects on microbial structure and activity in response to different stubble management strategies (slashed, slashed & incorporated, no stubble) with or without added chemical fertiliser.

Methods

Experimental setup: Soil profiles (0-10 cm) were recreated in tubs (41 x 64 x 28 cm) in a glasshouse assuming bulk densities of 1.2 and 1.1 g/cm^3 for Waikerie (Alfisol; organic C 0.6% and total N 0.055%, clay 3%) and Tarlee (Vertosol; organic C 1.5%, total N 0.16%, clay 43%) soils from the South Australian agricultural region, respectively. Wheat stubble, cut into segments ~ 5 cm long, was applied at rates equivalent to 3.5 and 4.8 t/ha for Waikerie and Tarlee soils, respectively; these rates represent dry matter yields seen in the field for each region. In the stubble incorporated treatment, stubble was incorporated to a depth of ~ 5 cm. After eleven months (5 months with weekly irrigation and 6 months undisturbed condition), stubble treatments were reintroduced. An additional treatment with fertiliser was added (@ 15 kg P and 40kg N and 20 kg P and 70kg N/ha, for Waikerie and Tarlee soils, respectively) and mixed to a depth of approximately 2 cm to ensure uniform distribution. All treatments were replicated four times. Tubs were maintained at constant temperatures of 20 °C and 12 °C (12h each) and water was applied weekly at rates representative of rainfall in the respective region. Four months after the addition of fertilizer, surface 0-5cm

soil was collected from a 2 x 5cm area in each tub and used to measure catabolic diversity and other biological properties. For molecular analysis, two 3cm dia. cores were collected from each tub, mixed and the entire soil sample (~50g) was used for DNA extraction (Ophel-Keller *et al.* 2008).

Catabolic diversity profiling: Measurement of the ability of soil microorganisms to utilize a diverse array of added C substrates provides a profile of microbial catabolic potential. Carbon substrate utilization profiles of soil microbial communities were determined using the Microresp® method (Campbell *et al.* 2003) modified with specific carbon substrates selected for Australian soils (Gupta VVSR, data unpublished). Catabolic potential for bacteria only was estimated by measuring C substrate use in soils amended with Captan, a broad spectrum fungicide.

Genetic diversity analysis (SSU Amplicon Pyrosequencing) of bacterial communities: Universal primers (~94.6% coverage) targeting the 16S rRNA gene was used for the initial amplification of soil genomic DNA. This allowed for multiple sample pyrosequencing on a single plate using the 454 Life Sciences FLX sequencing platform. Three independent replicate genomic DNA extractions and amplifications were performed per sample. Raw pyrosequencing reads were first filtered then aligned using INFERNAL 8.1 (Nawrocki and Eddy 2007) and a SSU rRNA secondary-structure model (Cannone *et al.* 2002). Sequences were then clustered by the complete-linkage method at the desired distance and assigned to specific taxa using the RDP Classifier (http://pyro.cme.msu.edu; Wang *et al.* 2007).

Data analysis: Multivariate statistical comparison of C-substrate utilization data was done using Genstat 12.1 (VSN International Ltd). Community level physiological profile (CLPP) analysis was used to differentiate microbial communities under different stubble and fertilizer treatments. For the DNA sequence data, the relationship among sites based solely on bacterial community structure was calculated using abundance-based adjusted Jaccard and Sorensen indices with EstimateS followed by hierarchical clustering (e.g. UPGMA tree construction). Indicator species analysis was used to identify specific clusters that significantly varied among treatments and between soil types.

Functional gene analysis: Nitrogenase reductase (*nif*H) gene fragments were amplified using primers described by Rösch *et al*. (2002) and quantified using the Stratagene Mx3000P qPCR system.

Results

Microbial biomass (MB) carbon levels ranged between 200 to 400µg C/g soil and accounted for 2-5% of soil organic C levels. Stubble retention increased MB C and N and microbial activity in both soils (data not presented) and the effect of fertilizer application was highest with stubble incorporation.

Table 1. Effect of fertilizer addition and stubble management on the catabolic potential of microbial communities (average CO_2 response for 22 substrates) measured using modified Microresp® methods.

Treatments		Tarlee (Vertosol)			Waikerie (Alfisol)		
		Total	**Bacteria[1]**	**Fungi[1]**	**Total**	**Bacteria**	**Fungi**
		µg CO2/g soil (5h assay)					
Without fertilizer	No Stubble	0.464	0.269	0.196	0.104	0.058	0.046
	Slashed	0.671			0.117		
	Incorp	0.729	0.352	0.377	0.158	0.056	0.103
	Standing	0.616			0.121		
With fertilizer	No Stubble	0.496	0.271	0.225	0.095	0.060	0.036
	Slashed	0.764			0.138		
	Incorp	0.807	0.386	0.421	0.211	0.058	0.153
	Standing	0.658			0.127		
Treat	**LSD (0.05)**	0.124	0.029	0.098	0.021	ns	0.033
Fert	**LSD (0.05)**	0.062			0.010		
Fert x Treat	**LSD (0.05)**	ns			0.021		

[1] CO_2 evolved from soils receiving the fungicide Captan prior to the C substrate utilization assay was considered as bacterial activity and the difference between total and Captan treated was attributed to soil fungi.

Catabolic diversity is a measure of the ability of microorganisms to utilize different types of C and N compounds and it reflects the functional capability of soil microbial communities as influenced by management and environmental factors. CLPP analysis showed significant differences between soil types and different stubble management treatments (data not shown). The effect of fertilizer addition on total

catabolic response was only observed in the presence of stubble (Table 1). Catabolic response was highest in the incorporated treatments followed by slashed and standing stubble treatments. Fertilizer application increased the contribution from fungi in the sandy soil but not in the Vertosol.

The number of *nif*H gene copies was higher in the Tarlee soil compared to that in Waikerie soil (1400 and 150 copies / nq DNA in Tarlee and Waikerie, respectively) and the trend was also reflected in N fixing potential (based on acetylene reduction (AR) bioassay). Stubble addition generally increased the *nif*H copy number and AR activity in both soils. Unlike the Vertosols, the sandy soil provides little opportunity for the development of stable microaggregates that can support free-living N_2-fixing bacterial communities (Hattori 1988).

Targeted 16S rRNA gene pyrosequencing resulted in a total of 113,895 16S rRNA gene sequences that were subjected to classification and clustering using the Ribosomal Database Project Pyrosequencing Pipeline (http://rdp.cme.msu.edu/). Unclassified bacteria accounted for 12,672 of the total at a 50% confidence threshold. Members of the phylum Actinobacteria were the most abundant (42%), followed by the Proteobacteria (21%), and the Acidobacteria (13%) (Figure 1). Complete linkage clustering at 5% operational taxonomic units (OTUs) resulted in 7467 clusters of which 2923 were classified as singletons that occurred in only one sample. Shannon and Chao indices showed that diversity increased in the Waikerie soil with stubble addition. Specifically, the number of unique clusters increased 60% with stubble incorporation at a 3% OTU level (Figure 2). Conversely, Tarlee soil diversity differed only marginally among treatments at all OTU levels.

Despite no clear influence of management regime, cluster-based UPGMA trees generated at both 3 and 5% OTU cutoff values with and without the inclusion of singletons showed that management effects influenced microbial community structure to some degree in the sandy Waikerie soil with no discernible effect in the Tarlee soil. Indicator species analysis was then undertaken in order to identify bacterial populations most responsive to treatment. Cluster dendogram branch lengths were notably smaller in the Tarlee soil as compared to the Waikerie soil. No significant indicator species were identified according to treatment type. However, using a false discovery rate, 48 highly significant ($q<0.003$) clusters were found that exhibited large differences between the two soil types. The phylum Actinobacteria accounted for 64% of the indicator species with a large proportion (>50%) belonging to the subclass Rubrobacteridae. Therefore, multiple independent modes of analysis showed that despite the application of identical treatments, soil type appears to have a dominate influence in determining bacterial community structure at these sites.

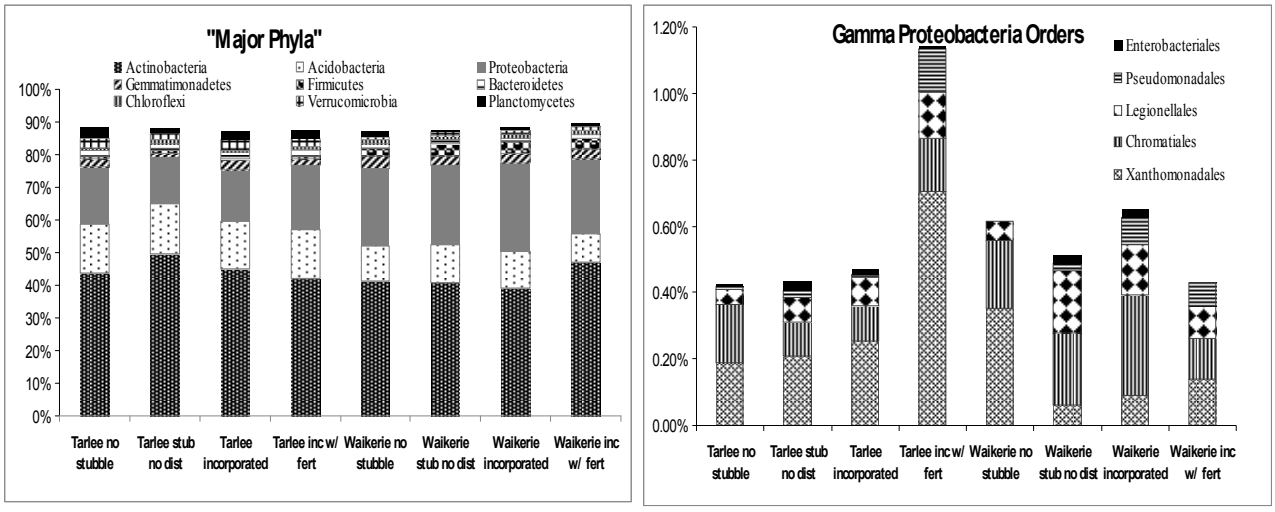

Figure 1. Effect of soil type and stubble management on the proportional distribution of bacterial taxa.

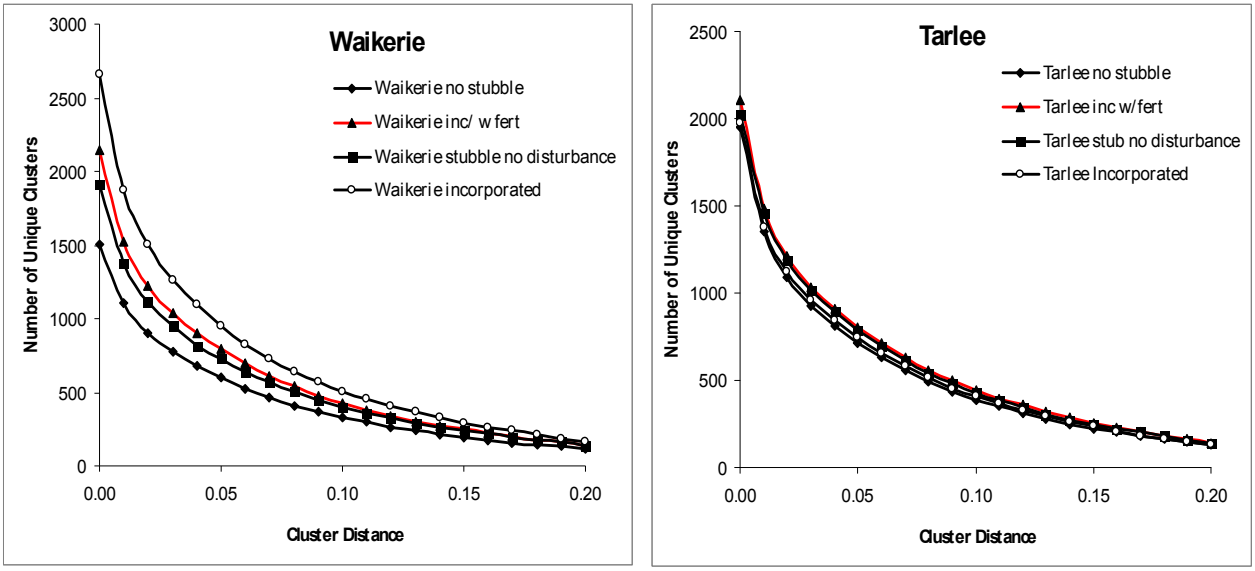

Figure 2. Changes in bacterial diversity as indicated by the number of unique clusters in the 16S sequences.

Conclusion

Stubble addition caused significant changes in the catabolic diversity of microbial communities in both soil types. Actinobacteria, Proteobacteria and Acidobacteria accounted for 76% of bacterial populations and bacterial community structure was different between soil types. The effect of stubble addition on the microbial diversity and activity, e.g. catabolic diversity of total microbial communities and genetic diversity of soil bacteria, was greatest when it was incorporated compared to standing stubble. Overall, differences in catabolic and genetic diversity of microbial communities can be attributed to the differences in the texture and chemical properties between soil types.

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Development of Soil Metadata Standards for International DNA Sequence Databases

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Abstract
The considerable growth in DNA sequencing and its application to microbial communities in many environments, including soils, has drawn researchers from many fields to soil microbial ecology and genomics studies. International convention is for all sequence data to be deposited in public databases as a community resource. However, for this data to be broadly useful information about the data, i.e. metadata, should also be deposited along with the sequence data. A subcommittee of Terragenome, a recently established international consortium to facilitate cooperation on soil metagenome studies, has developed a set of soil features important for understanding soil biology and for interpreting the sequence data. The committee has also defined an associated controlled vocabulary that will allow scientists to search the databases for sequence information that correlates with user defined environmental attributes. The work of the committee is summarized here and has been submitted to the Genomic Standards Consortium (GSC) so that it can be harmonized with data standards for other environments and be implemented as a GSC MIMS standard for use by the global scientific community.

Key Words
Metagenomics, Soil metadata, DNA sequencing, Microorganisms, Ribosomal RNA.

Introduction
Metagenomic studies generate huge amounts of sequence data; however, it is important to realize that these data are useful only within the context of the sample itself. This context is provided by metadata, which includes information necessary to repeat sample collection and preparation, and that describes the key factors that determine the composition of the microbial community and its function. Our efforts are extensions of metadata standards developed for ecology (Michener *et al.* 1997) and more recently for genomics to create minimum information checklists (http://darwin.nox.ac.uk/gc_wiki/index.php/GSC).

Methods
The attendees at the Terragenome II meeting in Lyon, France, in December 2008, endorsed a process of surveying the international community of soil biology researchers for the soil features that they thought would be important to understanding soil microbial community structure and activity, and that would be important to interpreting the molecular data entered into GenBank, EMBL and DDJB. This Web-based survey was available for respondents for approximately 4 months in the spring of 2008. Email address lists from several sources, including the international Terragenome contact list, were used to alert soil biologists of the request. The respondents were asked to evaluate a set of standard soil attributes for both their relative importance to understanding soil microbes and for the degree of difficulty in measuring that feature, the latter important for indicating likely compliance (Figure 1). One hundred five individuals from 15 countries responded. The summary information from this survey is at: http://cme.msu.edu/SoilMetadata/results.html.

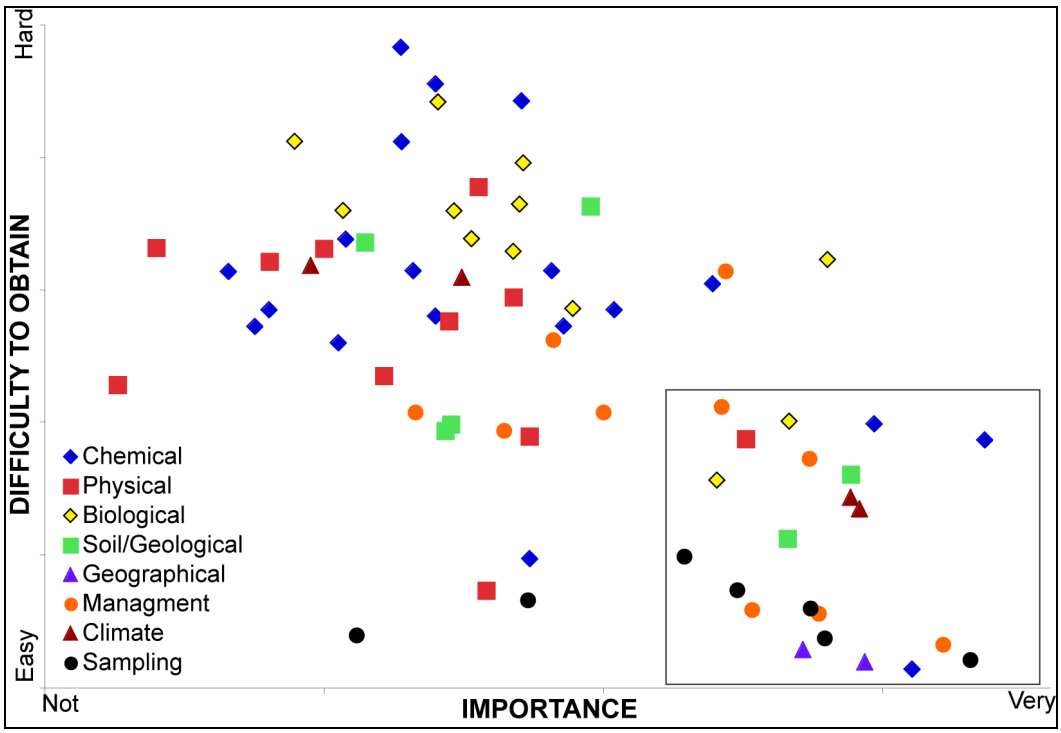


Figure 1. Results of Soil Metadata Survey with Importance and Difficulty displayed. Items in the box were the high priority items of focus in creating the data collection form. This graph can be viewed online [http://cme.msu.edu/jforum/SMSurvey.html] with mouse-over showing a description of each point.

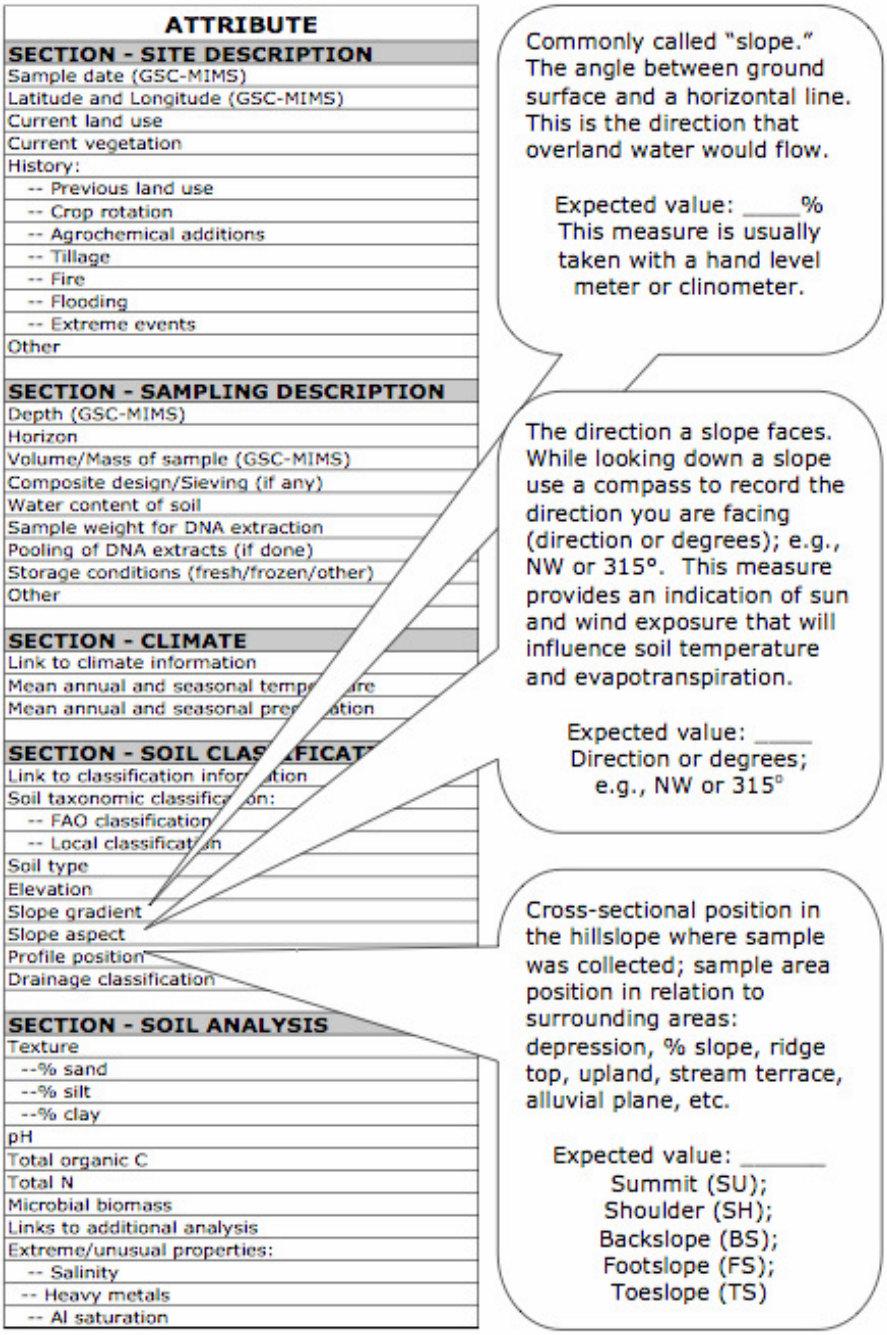
At Terragenome III in Uppsala in June 2009, a subcommittee was formed (the authors on this paper) to take the survey data and refine it into a priority set of features important to soil biology, organize it in a logical manner, develop a controlled vocabulary, provide a set of definitions for those not familiar with soil science terms and submit the recommendations to the international Genomic Standards Consortium. This has been done. The next stage is to work with database entry specialists to implement the metadata entry forms into a convenient and compliant tool. This is underway. The major primary sequence repositories as well as the metagenomic specialty databases such as CAMERA, MG_RAST and IMG/M will also be hosting these metadata.

Results

The structure of the proposed soil metagenome metadata consists of: (1) site description, (2) sampling description, (3) climate, (4) soil classification, and (5) soil analysis. Ideally, the metadata should be important and informative, easy and inexpensive to obtain, and collected by established and standard methods. The elements of the currently recommended data form are illustrated in Figure 2.

The site description documents the location and situation at the time of sample collection and also provides the information necessary to relocate the sampling site. The sampling description provides details related to the collection and processing of the sample, which would be required to repeat the analysis. Also included in these two categories is descriptive information related to the past and current status of the site, which may be useful in interpreting the metagenomic data or ensuring that similar conditions exist if a site is re-sampled.

Figure 2. Soil attribute form. Users can mouse over the attribute name and view a pop-up with the definition and expected value (accepted units). Drop-down selections, such as illustrated, provide the primer for features less known to the diverse user community.



The remaining three categories provide data about the environmental and edaphic factors that are likely to influence the composition of the microbial community. Environmental factors include basic information about the site's climate, which can likely be obtained from the historical records of nearby weather stations. Soil classification and analysis were separated based on the idea that the classification information is integrative in nature, reflecting the soil forming factors acting at a given site. They are descriptive (i.e., qualitative) in nature. The soil analysis data are quantitative and reflect soil properties that are likely to be most significant regulators of microbial community composition.

A primer was also developed to aid this diverse community of scientists we expect will be depositing soil metadata. There were multiple purposes for the primer. (1) To recognize the international differences in terms and definitions used in soil science. (2) To provide some guidance on the expected metadata to be deposited. (3) To provide some simple definitions for those who have not had formal training in soil science. (4) To provide some guidance on measures that can be taken at the time of soil sampling. (5) To provide

some web resources that can be used to obtain more in depth information if desired. The primer was adapted from information published by two major sources, the Schoeneberger *et al.* (2002) soils field guide and U.S. Department of Agriculture-Natural Resources Conservation Service (USDA-NRCS) soil survey manual. Information was cross-checked with any available soil resources from other countries and international societies.

The major topics covered in the primer are soil property measures and land use descriptions. We expect most of the metadata will be easily deposited using drop-down menus, such as illustrated in Figure 2 for a particular item. The soil properties defined in the primer are: slope, drainage, horizon, and texture. Definitions are also provided for land use in general and specific agricultural tillage treatment of soils. The current data form is at: http://cme.msu.edu/SoilMetadata/SoilMetadataEntryTEMPLATE.xls

Conclusions

Metadata will help place annotated soil metagenome data into their correct context. By submitting these standards to the GSC for potential inclusion in the MIMS (Minimal Information about a Metagenome Sequence) standard (Field *et al.* 2008), these standards will reach a larger audience and be incorporated in data preparation and search tools supporting the GSC standards. Researchers from many fields -- e.g., marine biology, human microbiome, environmental engineering, as well as soil science -- will be able to query and retrieve metagenome data from all studied microbial communities based on their metadata content. This will facilitate comparative studies and allow future researchers to incorporate today's data into future integrative studies and advance our understanding of soil microbiology in ways that we may not be able to foresee today.

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Ecology of viruses in rice fields

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Abstract
Viruses are the most abundant biological entities in marine and freshwater environments. Many studies have shown the ecological importance of viruses as the greatest genomic reservoirs, in primary production and microbial food web in aquatic environments. However, the viral ecology in the soil environment has been little documented. In this study, we estimated viral abundance in the floodwater of rice fields, and then measured phage-infected bacterial cells for evaluating the importance of phage infection to bacterial hosts in the microbial food web. The diversity and specificity of *g23* sequences of T4-type bacteriophages in rice fields were also determined in comparison with those in aquatic environments. Viruses were also the most abundant biological entities in rice fields, and bacteriophages comprised the majority among viral communities. The high frequency of phage-infected bacterial cells indicated that the bacterial mortality from phage lysis could be significant enough to redirect the microbial food web and change the bacterial communities. The majority of *g23* sequences of T4-type bacteriophages in rice fields were distantly related to those of marine origins. The *g23* genes in rice fields have apparently diverged more compared to marine *g23* genes.

Key Words
Phage, *g23* gene, abundance, phage-infected bacterial cell, diversity, morphology

Introduction

Viruses are the most abundant biological entities in marine and freshwater environments. Many studies have indicated the ecological importance of viruses in global biogeochemical nutrient cycles. Viruses are recognised as the greatest genomic reservoirs in marine and freshwater environments due to their huge abundance and diversity. However, to date the ecology of soil viruses has been studied little at the community level.

We studied the seasonal variation in the abundance of viruses and bacteria in the floodwater of a rice field. Then, as phages comprised the majority of viral communities in the floodwater, the frequency of phage-infected bacterial cells in the floodwater was measured to evaluate the importance of phage communities in the microbial food web in rice fields. From the analysis of the capsid gene, *g23*, of the T4-type phages in rice field soils in Japan and Northeast China, many novel *g23* gene sequences were detected, which suggested the presence of soil-specific phage communities. These findings indicated the importance of viruses as genomic reservoirs and in the microbial food web in rice fields.

Materials and Methods

Enumeration of virus-like particles (VLPs) and their morphology
Enumeration of virus-like particles (VLPs) was performed by epifluorescence microscopy (EFM) after staining with SYBR Green I for the floodwaters collected from a Japanese rice field. Then the morphology of viruses was examined by transmission electron microscopy (TEM) for the same floodwater samples.

Frequency of visibly infected bacterial cells (FVIC)
The bacterial mortality in the floodwater due to phage lysis was evaluated by measuring the frequency of phage-infected bacterial cells with a transmission electron microscope (TEM). Floodwater samples were the same samples used for studying the viral abundance. The frequency of visibly infected bacterial cells (FVIC) was calculated from the proportion of phage-infected cells in the total number of cells examined.

Phylogeny and diversity of major capsid gene g23 *in rice fields*
To evaluate the phylogenetic characteristics and diversity of phages in rice field soils, *g23* genes of T4-type phages, *Myoviridae*, were PCR-amplified and sequenced from DNA extracted from rice field soils in Japan and Northeast China. *g23* genes in the floodwater were also studied by using the same floodwater samples mentioned above. The primers used for the *g23* amplification were MZIA1bis and MZIA6 (Filée *et al.* 2005). Denaturing gradient gel electrophoresis (DGGE) was conducted to the PCR products to efficiently obtain different *g23* clones.

Results and Discussion
Abundance and morphology of viruses in the floodwater
Viruses are the most abundant biological entities in the floodwater of rice fields. VLP abundance in the floodwater during rice cultivation period ranged from 5.6×10^6 to 1.2×10^9 VLPs/mL (mean abundance of 1.5×10^8 VLPs/mL). In contrast, VLP in the irrigation water ranged from 5.1×10^6 to 1.1×10^7 VLPs/mL. Smaller VLP abundance in the irrigation water than that in the floodwater indicated that viruses in the floodwater were not derived directly from the irrigation water but occurred in the floodwater. The high VLP abundance in the floodwater suggested that the floodwater is an aquatic environment abundant in viruses in comparison with marine, estuarine, and freshwater environments, where VLP abundance is generally less than 10^7 VLPs/mL (Wommack and Colwell 2000; Weinbauer 2004).

The virus-to-bacterium ratio (VBR) in the floodwater ranged from 0.11 to 72. VLP abundance showed significant correlation with bacterial abundance ($P < 0.01$). Although there was no significant correlation between VBR and VLP abundance, seasonal variation in VBR correlated significantly with the change of bacterial abundance, and VBR increased with the decrease in bacterial abundance ($P < 0.01$).

The capsid size distribution was monophasic, and the median values fell into the 50-60 nm or 60-70 nm fraction. More than half of the viruses fell within the size ranges from either 40-50 nm or 50-60 nm to either 60-70 nm or 70-80 nm for most samples. The predominance of viruses with the size classes of <100 nm was attributed to the predominance of phages in viral communities because the average capsid size for viruses of eukaryotic algae is reported to be 152 nm (Van Etten *et al.* 1991).

The most common capsid forms were tailed or non-tailed, isometric icosahedral form with a diameter of 50-70 nm, and other forms were too rare and sporadic to evaluate the difference in diversity among sampling dates from the morphology. Dominant viruses were estimated to be myoviruses (with a long contractile tail), siphoviruses (with a long non-contractile tail), and podoviruses (with a short non-contractile tail). Rough estimation of tailed viruses by TEM accounted for 2 to 54 % of the total in this study. As Ackermann (2001) summarised that 96% of phages were tailed among ca. 5,100 phages examined by TEM, the sonication used in our sample preparation might have caused tail loss in a considerable proportion of the viruses.

Frequency of phage-infected bacterial cells (FIC) in the floodwater
Weinbauer (2004) reviewed the virus-mediated carbon flow in pelagic oceans, where 6 to 26% of the carbon fixed by primary producers enters into the dissolved organic carbon (DOC) pool via virus-induced lysis at different trophic levels.

The FVIC ranged from 1.6 to 3.6 % for the floodwater samples. By TEM, phage particles are recognisable in the infected cells only in the late stage of the lytic cycle. Therefore, the frequency of phage-infected bacterial cells (FIC) was estimated from the FVIC by considering the fraction of the eclipse period in the latent period. The FIC ranged from 10.5% to 22.5 %, and the fraction of bacterial mortality from phage lysis was estimated to range from 12.8 to 35.0 % according to the non-linear steady-state model of Binder (1999). The frequency of phage lysis in floodwater was within the frequency ranges observed in other aquatic environments (Weinbauer 2004). The impact on bacterial mortality by phage lysis seemed to be large enough to redirect the microbial food web and induce the change and succession of bacterial communities in the floodwater since phages are, in general, strictly host-specific.

Characteristics and diversity of phages in rice field soils – estimation by g23 gene sequences of T4-type phages

Deduced amino acid residues of the partial *g23* fragments were aligned first together with the representatives of T-evens, PseudoT-evens, SchizoT-evens and ExoT-evens as well as marine *g23* clones. The majority of soil *g23* sequences were distantly related to the T-evens sequences and those of marine origin and formed several independent clusters. Thus, T4-type phage communities in rice fields consisted of previously uncharacterised members phylogenetically distant from those found in marine environments.

The neighbour-joining phylogenetic tree showed the close relationships of *g23* amino acid sequences from Chinese *g23* clones with those obtained from Japanese rice fields. The clones/phages formed nine clusters (Paddy Groups I to IX) with small clusters of ungrouped paddy clones. Many clones in Northeast China shared groupings with the clones in Japanese rice fields while also showing one Chinese-specific group. In general, there was no tendency for the clones from the specific types of soil and fertilisers, the stage during rice cultivation, or the location, at least within Japan or Northeast China, to belong to the specific Paddy Groups, which indicated that they were not the major factors in determining T4-type phage communities.

Short and Suttle (2005) reported that nearly identical (>99%) sequences of another major capsid gene, *g20*, of phages were recovered from marine and freshwater environments, suggesting that either closely related hosts and the viruses infecting them were distributed widely across environments, or horizontal gene exchange occurs among phage communities in very different environments. Similar clues suggesting the horizontal gene exchange/transfer were obtained in the *g23* sequence analysis. Firstly the identical *g23* sequences at the nucleotide level were observed in two cases among the phages having genome sizes of 60 kb and 160 kb as well as among some phages with isometric and elongated capsids. Furthermore, identical *g23* sequences at the nucleotide level were also observed at distant rice fields. In addition, several cases of identical *g23* sequences at the nucleotide level were observed among the clones obtained from rice fields in Japan and China.

The unrooted phylogenetic tree demonstrated that the majority of *g23* fragments from rice fields (soils and floodwater) formed several clusters independent from those derived from marine environments. The marine groups were distributed narrowly in the tree, which indicated that *g23* genes in paddy fields are more divergent in comparison with those in the marine environment.

In addition, the study on soil depth profiles of *g23* clones to a depth of 1 m indicated that T4-type phage communities of rooting layers were different from those of subsoil layers. This finding suggested that root development may change eubacterial communities in the rooting layers, with resultant changes in *g23* composition (T4-type phage communities). Many uncharacterised *g23* genes and their large diversification as described in this study strongly suggested that rice fields are great genomic reservoirs of viruses.

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Functional proteome analysis of wheat: systematic classification of abiotic stress-responsive proteins

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Abstract
Crucial functions of the plant cell are to take action against environmental stresses for self-defense. Evaluation of two-dimensional electrophoresis gels revealed several proteins to be differentially expressed as a result of abiotic stress among cultivars. 217 protein spots of interest were, after an in-gel tryptic digestion, identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Ten percent of abiotic stress responsive proteins were identified in cv. Keumgang followed by 7% in cv. Jinpum and cv. China-108, 12% in cv. Yeonnon-78, 31% in cv. Norin-61 and 33% in cv. Kantou-107 in our experiment. Of the total number of 575 identified proteins, 345 proteins were recognized as abiotic stress responses unique proteins with isoforms, of which 34% are induced by heat, 27% by drought, 15% by salt, 13% by cold and 11% by other environmental stress. Furthermore, elucidating the function of proteins expressed by genes in stress tolerant and susceptible plants will not only advance our understanding of plant adaptation and tolerance to environmental stresses, but also may provide important information for designing new strategies for crop improvement.

Key Words
Abiotic stress, matrix-assisted laser desorption/ionization-time of flight, proteomics, wheat.

Introduction
Any living organism has to survive with conditions of stress. Specifically for plants, the possibilities to escape from stress are limited because plants are motionless (Kuiper 1998). As a general rule, emblematic response to environmental stress conditions is established by the induction of a set of stress proteins that protects the organism from cellular damage. Abiotic stresses such as heat, cold, drought, salinity, nutrient, ozone, heavy metals, ultra-violet light, visible light, chemical toxicity and oxidative stress are serious intimidation to agriculture. In this study, we determined specific proteins induced by each abiotic stress, particular emphasis will be placed on the heat shock, drought, cold, salt and others environmental stress by using proteomic approaches, which is fruitful information for improving stress tolerance cultivars.

Materials and methods
Plant materials
Six genotype of wheat seeds (two Korean: Keumgang, Jinpum; two Chinese: China-108, Yennon-78 and two Japanese: Norin-61, Kantou-107) were used in this study for identification of biotic stress responses proteins by proteomics analysis.

Sample preparation by KCl solubility method
Osborne's (1924) solubility method that we routinely use to fractionate wheat endosperm proteins takes advantage of the solubility properties of wheat endosperm proteins in KCl, SDS, and acetone with some modifications (Hurkman and Tanaka 2007).

Two-dimensional gel electrophoresis (2-DE)
Soluble proteins of whole seed storage were examined by two-dimensional gel electrophoresis (Kamal et al 2009a,b). Protein spots in 2-DE gels were visualized by Coomassie Brilliant Blue (CBB) R-250 staining (Woo *et al.* 2002). Each sample was run three times and the best visualized gels were selected.

In-gel digestion and mass spectrometry analysis
Selected protein spots were excised from preparative loaded gels, stained with Coomassie brilliant blue (R-

250), then washed with 100 µl distilled water couple to tryptic digestion (Kamal *et al.* 2009a,b). In MALDI-TOF/MS (AXIMA CFR$^+$ Plus, Shimadzu, Japan) analysis, proteins separated by 2-DE were digested in gels according to the method described by Fukuda *et al.* (2003).

Bioinformatics analysis

The proteins were identified by searching NCBInr, SWISSPORT, MASCOT database using the MASCOT program (http://www.matrixscience.com,Matrixscienc,UK). When more than one peptide sequence was assigned to a spectrum with a significant score, the spectra were manually examined. Sequence length, gene name and also protein functions were identified by searching Swiss-Prot / TrEMBL database using UniProtKB (http://www.uniprot.org).

Results and discussion

We observed heat increases or decreases in proteins by heat stress using proteomic technique in wheat grain. These proteins include heat shock proteins (1,000-91,000), heat stress transcription factor (27,000-54,000), granule bound starch synthesis (58,000-60,000), GTP binding proteins (22,000-24,000), beta-amylase (9,000-24,000), eucaryotic initaition factor (12,000-46,000), elongation factor (24,000-50,000), ribulose biphosphate (13,000-52,000) related proteins and so on, which are found in mature seeds. These results confirmed the results previously by Majoul *et al.* 2004, demonstrating that the synthesis of HSPs occurs in the full range of wheat tissues including developing grains (Giornini and Galili 1991). These drought adaptive changes rely largely on alterations in gene expression. We identified different abscisic acid responsive proteins (10,000-41,000), LEA protein (12,000) such as dehydrins (16,000-30,000), chaperonin (10,000), cys peroxiredoxin (23,000-24,000), ethylene response (19,000-28,000), and elongation factor TU (50,000) in six wheat cultivars, which is responsible for drought stress.

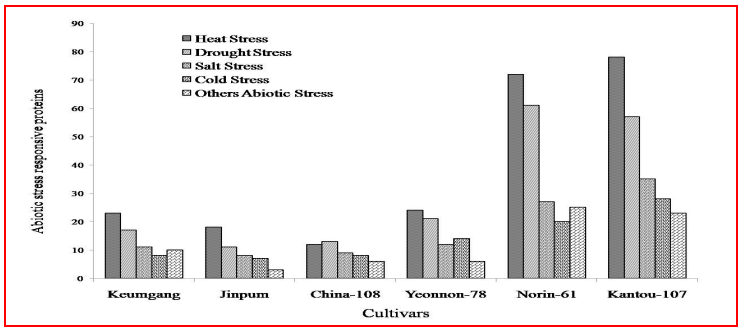

Figure 1. Functional distribution of the total identified abiotic stress responsive proteins among wheat cultivars.

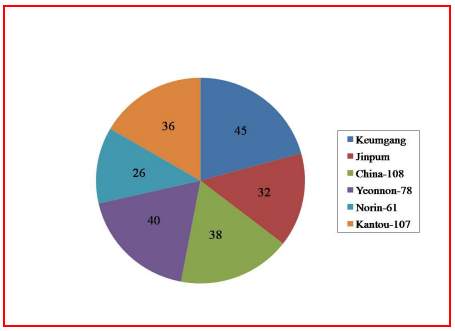

Figure 2. Distribution of total detected protein spots by two-dimensional electrophoresis.

The plants show sorts of adaptation in any specific temperature. Plants face high and low-temperature stress. In our experiment, we revealed some cold stress related proteins such as cold acclimation proteins (9,000-22,000), cold shock proteins (16,000-38,000), ABA inducible proteins(10,000-41,000), cyclophilin (13,000-18,000), low temperature regulated proteins (7,000-14,000), kinase like protein (6,000-74,000), mitogen activated (40,000-80,000), transcriptional adaptor (7,000-29,000), and translation initiation (12,000-17,000) in six wheat cultivars. These results would confirm previous work describing to determine the genetic nature of these mechanisms. Several cold-responsive genes of unknown function were identified from cold-acclimated wheat (Breton *et al.* 2003). The most common plant stress in soils is salinity. Some aspects of salt stress responses are intimately related to drought and cold stress responses (Zhu 2001). In our experiment, we identified some salt stress responsive proteins such as salt stress protein (8,000-26,000), ABA inducible (10,000-41,000), aquaporin (21,000-31,000), peroxiredoxin (23,000-24,000), bowman-brik type proteinase inhibitor (5,000), calcineurin B like protein (25,000-29,000), cyclophilin (1,000-22,000), zinc finer protein (3,000-43,000), potassium channel (81,000-93,000), calcium channel (8,000), and RNA binding proteins (16,000-20,000) in six wheat. Two-dimensional electrophoresis was used to reveal changes in protein expression of rice; they identified several salt responsive proteins including salt stress protein and ABA (Dooki *et al.* 2006). We identified heavy metals such as cadmium (18,000), copper (14,000-19,000), aluminium (49,000), manganese (19,000), metallothion like (4,000-42,000), molybdenum (20,000), Rab GTP (guanine nucleotide-binding proteins) binding (2,000), Rac/Ras like GTP binding (23,000-24,000),

germin like (23,000-24,000) wall associated kinase (57,000-88,000), and some unclear abiotic stress responsive proteins in our experiments.

Conclusion

Using two-dimensional electrophoresis, this study identified proteins involved in heat, drought, cold, salt and some others abiotic stress responses in wheat. Our findings reveal a proteomic profile of abiotic stress in wheat, which may provide benefits in two major areas, in the better understanding of abiotic stress proteins including their functions, and the understanding of stress related physiology in wheat grain.

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Gene expression and proteomics in soil

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Abstract
Molecular techniques have mainly been applied to soil to characterise DNA so as to assess microbial diversity whereas few studies have been carried out to monitor gene expression by extraction and characterization of mRNA and proteins. Novel techniques are available to extract mRNA from soil with successive characterisation. Functional metagenomics based on cloning of large genomic inserts containing operons and promoters can allow screening for specific functions and novel bioproducts. Soil proteomics can give useful information on cell-environment processes, by analysing proteins acting as biosensors, extracellular enzyme activities, stress proteins and metabolic proteins, and on homologous and heterologous cell-to cell interactions with analysis of proteins or peptides involved in quorum sensing and genetic exchange activities, or defining competition, predation, commensalims and symbiosis.

Key Words
Microbial diversity, genomics, proteomics, soil functionality.

Molecular techniques for detecting DNA sequences have been extensively used in soil and with metagenomic technologies it is possible to improve the determination of composition of complex microbial communities such as those inhabiting soil (Urich *et al*. 2008). However these DNA-based studies do not allow determining gene expression in soil and thus are not indicative of soil processes. Monitoring the expression of gene sequences at both transcription and translational levels is needed in soil science in analogy to post genomic studies of cultivated organisms. The analysis of the target mRNA is supposed to be an indicator of the transcription activity in soil. Despite the fact that extraction procedures of soil RNA and DNA are similar in principle, successful extraction and characterization of mRNA from soil has lagged behind those of DNA due to problems such as activity of nucleases and fast turnover rate of prokaryotic mRNA (Costa *et al*. 2004; Bakken and Frostegard 2006). However, several methods are now available to characterise mRNA and thus to measure gene expression in soil (Metcalfe *et al*. 2002; Krsek *et al.* 2006). An RNA meta-trascriptome approach involving the extraction of both mRNA and rRNA from a sandy soil, with reverse transcription to cDNA and with direct pyrosequencing, produced both cDNArRNA-tags and m-RNA-tags and this allowed the quantification of abundant microorganisms and information of the activity of enzymes involved in ammonia oxidation and CO_2 fixation (Urich *et al*. 2008).

Multiple protein isoforms can be synthesised by a single gene because mRNA molecules can be subjected to post-transcriptional control such as alternative splicing, polyadenylation and mRNA editing (Graves and Haystead 2002). The analysis of expressed proteins in pure culture is rapid and sensitive and involves extraction of proteins, their separation by 2-dimensional gel, the solubilisation of excised band and successive trypsin digestion followed by analysis of tryptic peptides by ionization mass spectrometers (Pandy and Mann 2000). The use of bioinformatics for processing a huge mass of data is also required.

The application of proteomic studies to soil should consider that on average microbial N accounts for 4% of organic N in soil whereas most of the total organic N is present as extracellular protein N or peptides N stabilised by soil colloids (Nannipieri 2006). In addition, information on microbial gene expression related to microbial activity should be based on the characterization of intracellular microbial proteins unless information on microbial processes involving extracellular enzymes is required. The characterization of extracellular proteins, protected against proteolysis by their association with soil colloids, should give insights on mechanisms responsible of such stabilization. The two approaches have been termed soil functional proteomics and soil structural proteomics, respectively (Nannipieri 2006). The study of functional proteomics can improve our understanding of degradation of organic pollutants and organic debris, nutrient cycling, blockage of inorganic pollutants, molecular colloquia between microorganisms, between plant roots and microorganisms and between plant roots (Nannipieri 2006). A successful extraction of intracellular proteins from soil should lyse microbial cells, inhibit proteases, avoid the adsorption of proteins with soil colloids once released after cell lysis and be representative of the status of microorganisms inhabiting soil. The manipulation of the sample prior to extraction should not alter microbial physiology.

Most of the reports on soil proteomics are based on the direct extraction method (Nannipieri 2006). Caution is required in using both colorimetric (Bradford method) or immunological (ELISA) methods to determine extracted proteins since these techniques can also determine phenolic compounds and litter and humic components (Rosier *et al*. 2006; Whiffen *et al*. 2007; Roberts and Jones 2008).

Proteins have been extracted from dissolved organic matter of forest soil, purified by gel filtration, with removal of humic acids, phenolic compounds and small molecules, and concentrated by ethanol before SDS-PAGE (Schulze 2004). After silver staining, each protein band was cut, digested by trypsin and mixtures of tryptic peptides separated by nanoflow liquid chromatography prior to analysis by mass spectrometry. The phylogenetic origin of proteins was evaluated and bacterial proteins were classified in ribosomal, transcription, membrane and enzyme proteins according to their function. The power of analytical MS tool was also shown by detecting chlorocatechol dioxygenases, enzymes involved in the degradation of 2,4-D (2,4-dichlorophenoxy acetic acid), after mass spectrometry analysis of tryptic peptides obtained from excised bands of SDS-PAGE gels, obtained by electrophoresis of protein preparations extracted by 0.1 M NaOH from 2,4-D treated soils and successively purified (Benndorf *et al*. 2007).

As it is done for the extraction of nucleic acids from soil, separation of microbial cells from soil particles and successive cell lysis with release of proteins is an alternative approach to extract microbial proteins from soil. The microbial extraction prior to cell lysis should give purer samples but with lower yields than the direct extraction method. However, artefacts due to possible changes in the physiology of microorganisms can occur during the extraction of microbial cells from soil (Nannipieri 2006) making it difficult to relate the microbial proteins to the effects of the studied factors (i.e. stresses, agriculture management, etc). Maron *et al*. (2008) used the indirect extraction method to show that copper or mercury pollution of soil stimulated the synthesis of protein with molecular weight ranging from 20 to 50 kDa; some of these proteins, such as those of heavy metal efflux pumps, were involved in heavy metal resistance mechanisms.

Phosphate is more efficient than other buffers in extracting proteins from soil. However each extraction procedure should be tested with a microbial strain with a known proteome so as to evaluate any effect of the used procedure on the microbial proteome. We have tested our extraction procedure on *Cupriavidus metallidurans* CH34.

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Glucose effects on denitrifier abundance, denitrification gene mrna levels, and denitrification activity in an anoxic soil microcosm

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Abstract
Organic carbon availability influences nitrous oxide (N_2O) emissions but its effect on denitrifier communities is not understood. Changes in denitrifier abundance, denitrification gene mRNA levels and denitrification activity were followed in anoxic soil microcosms in the presence and absence of glucose with non-limiting nitrate concentration for 48 h. *nosZ* and *nirS$_p$* (*Pseudomonas mandelii* and closely-related spp.) genes (qPCR) and mRNA levels (qRT-PCR) were quantified. Abundance of *nosZ* and *nirS$_p$* were unaffected by glucose addition and were stable over the duration of the incubation with average values of 4.3 x10^8 and 8.1 x10^4 gene number/g dry soil, respectively. *nirS$_p$* mRNA levels were increased by glucose addition. Glucose addition resulted in induction of *nirS$_p$* mRNA levels after 4 h, with a 2.5 fold increase in transcripts compared with 0 h, to 2.4 x10^4 transcripts /g dry soil. In contrast, *nosZ* mRNA levels were not affected by glucose addition and averaged 2.3 x10^6 transcripts /g dry soil. Glucose addition increased cumulative N_2O emissions, with final values of 4.9 and 0.9 mg N_2O-N /kg dry soil for the glucose amended and non-amended soils, respectively, at 48 hr. The increase in N_2O emissions resulting from glucose addition in this study were not clearly accompanied by significant changes in abundance or denitrification gene mRNA levels for the targeted bacterial communities.

Key Words
nosZ, nirS, quantitative PCR, gene expression, organic carbon, *Pseudomonas mandelii.*

Introduction
Denitrification, the dissimilatory reduction of nitrogen oxides, is a metabolic process performed by soil bacteria that produces the greenhouse gas nitrous oxide (N_2O) as an intermediate gaseous product. Denitrification is influenced by environmental factors including oxygen concentration and nitrate concentrations; however, carbon availability is likely one of the most important factors influencing denitrification (Miller *et al.* 2008). Organic carbon addition reduces soil oxygen supply by promoting microbial growth, favouring the denitrification process. Organic carbon is also used as an electron donor in denitrification (Zumft 1997). Glucose, a simple carbon source, was used in several denitrification studies (Dandie *et al.* 2007, Fischer *et al.* 2005, Miller *et al.* 2008, Murray *et al.* 2004) and was used in this study as a first step in a larger project to understand the relationships between denitrifier activity and organic carbon sources in agricultural soils. The objective of this study was to evaluate the effect of a simple carbon source, glucose, on denitrifier abundance, denitrification gene mRNA levels, and denitrification activity from an agricultural soil. We hypothesized that an increase in denitrification activity, after the addition of glucose, would be due to an increase in the abundance of denitrifiers and/or the abundance of denitrification gene transcripts.

Methods
Experimental Design
Treatments with or without glucose addition (0 or 500 mg glucose-C /kg dry soil) were applied to soil cores in a factorial arrangement (2 levels of glucose, 6 incubation times, 4 replicates) in a completely randomised design. Nitrate was added at 500 mg NO_3-N/g dry soil (as KNO_3) and all cores were incubated at 70% water-filled pore space in sealed jars. Two sets of jars were used to measure denitrification and N_2O emissions. Cumulative denitrification was measured in one set of jars by adding acetylene (C_2H_2) to the headspace to block N_2O reduction ($N_2O + N_2$). No C_2H_2 was added to a second set of jars to measure N_2O emissions. Headspace gas samples (20 mL) were taken using a syringe. Soils were destructively sampled at

0, 4, 8, 12, 24, and 48 h of incubation. For nucleic acid extractions, soil samples were flash frozen in liquid nitrogen immediately after sampling and stored at -80°C until processing.

Analyses and statistics

Extractable organic carbon (EOC) and NO_3^- concentrations were measured in K_2SO_4 extracts from soil samples using colorimetric analysis on a Technicon Auto Analyzer II system (Technicon Industrial Systems, Terrytown, MA, USA). Headspace gas was analyzed for N_2O and CO_2 concentrations using a Varian Star 3800 Gas Chromatograph (Varian, Walnut Creek, CA) fitted with an electron capture detector (to measure N_2O), thermal conductivity detector (to measure CO_2), and a Combi-PAL Autosampler (CTC Analytics, Zwingen, Switzerland) (Burton *et al.* 2008). Nucleic acids (DNA and RNA) were extracted from freeze-dried soil samples using methods adapted from Griffiths *et al.* (2000).

Denitrification genes and transcripts (*nosZ* and *nirS*$_p$) were quantified via qPCR and qRT-PCR using an Applied Biosystems (Streetsville, ON) ABI PRISM® 7000 thermal cycler and SYBR Green detection as described in Henderson *et al.* 2010. ANOVA was performed with treatment and time as fixed factors. Means comparisons were performed for significant main effects and interactions by performing post hoc Tukey HSD and Tukey adjusted LS means, respectively.

Results and discussion

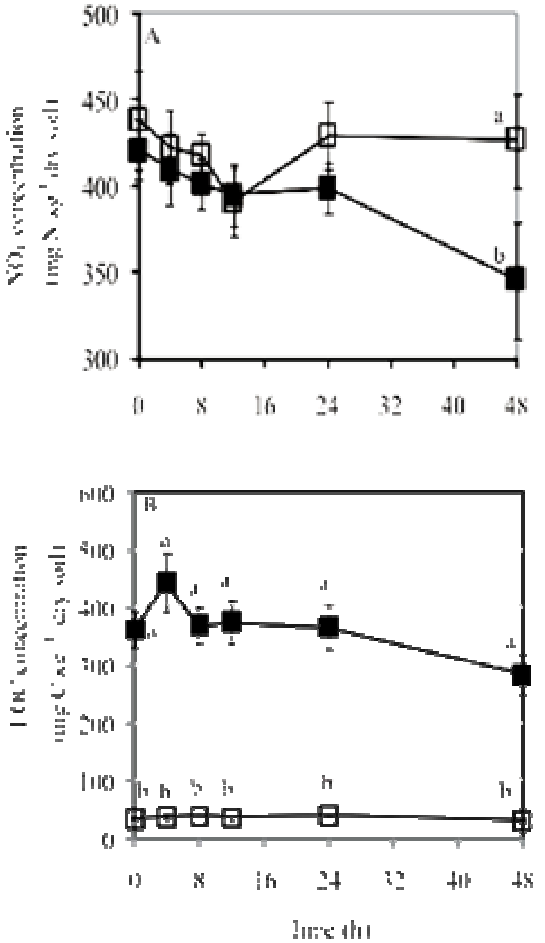

Figure 1. Soil concentrations of nitrate (NO_3^-) (A) and extractable organic carbon (EOC) (B) for soil incubated over 48 h following addition of glucose at 0 mg C-glucose /kg dry soil (G0) (□) or 500 mg C-glucose /kg dry soil (G500) (■). Values are means ± SEM (*n*=4).

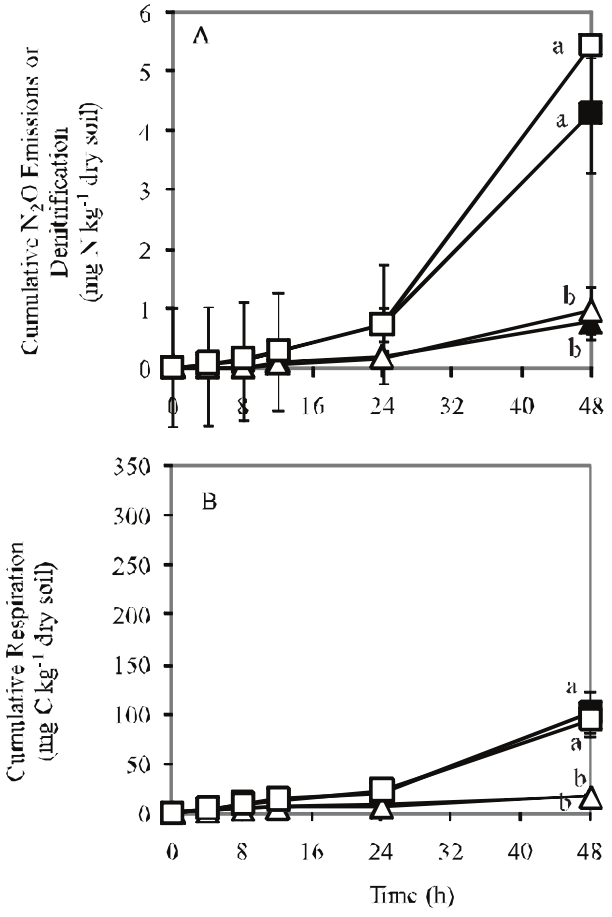

Figure 2. Cumulative emissions of nitrous oxide (N_2O) and denitrification (i.e. $N_2O + N_2$) (A) and carbon dioxide (CO_2) (respiration) (B) from soil incubated over 48 h following addition of glucose at 0 mg C-glucose/kg dry soil (G0) with (▲) or without (△) C_2H_2, or addition of 500 mg C-glucose/kg dry soil (G500) with (■) or without (□) C_2H_2. Values are means ± SEM (*n*=4).

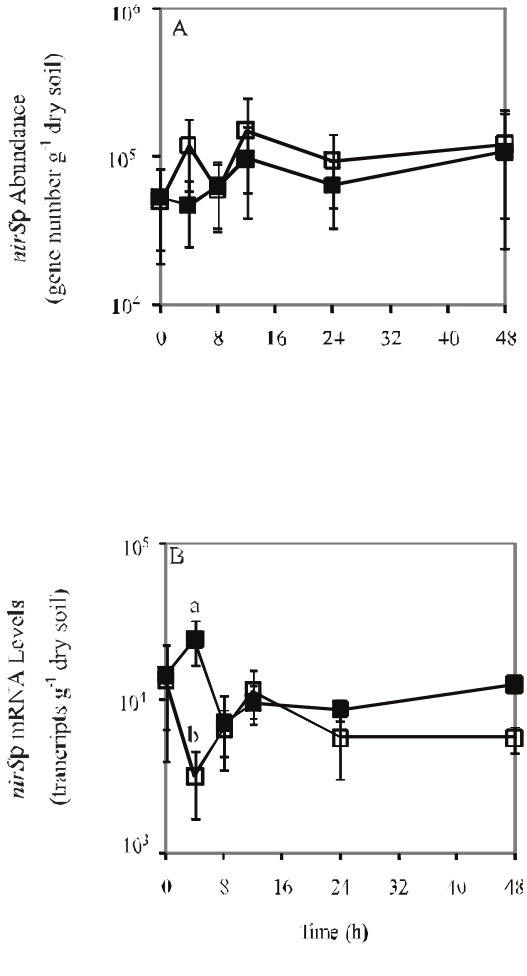

Figure 3. Quantification of *nirS$_p$* gene abundance using qPCR (A) and *nirS$_p$* mRNA levels using qRT-PCR (B) in soil incubated over 48 h following addition of glucose at 0 (G0) (□) or 500 mg C-glucose /kg dry soil (G500) (■). Standard curve descriptors of quantification of *nirS$_p$* gene numbers and detection levels: y = -3.39x + 36.0, R^2 = 0.998, E = 97.1%, no template control (NTC) = undetected. Standard curve descriptors of quantification of *nirS$_p$* transcripts and detection levels: y = -3.43x + 36.3, R^2 = 0.994, E = 95.3%, NTC = undetected. Values are means ± SEM (*n*=4).

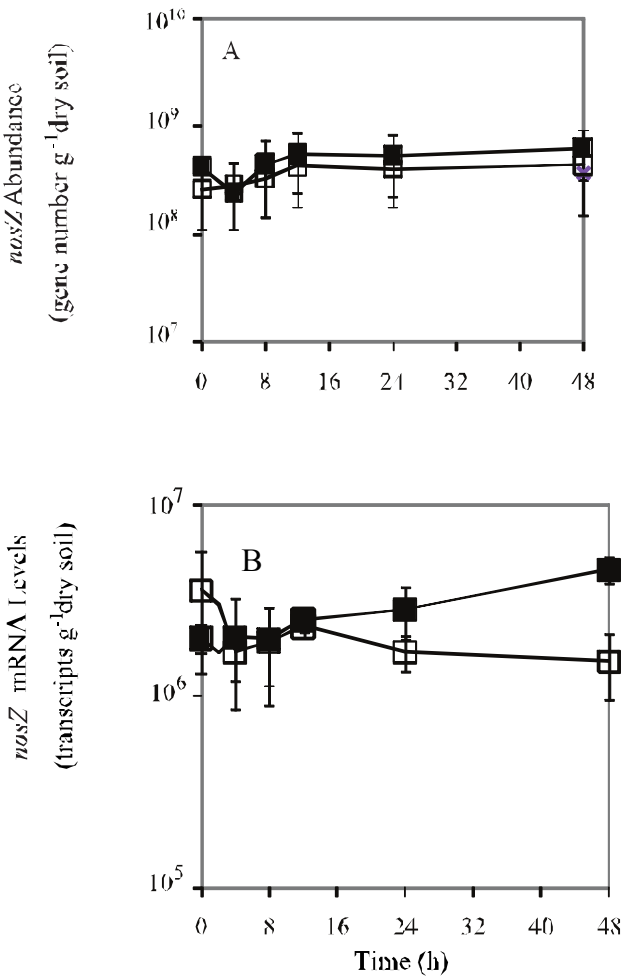

Figure 4. Quantification of *nosZ* gene abundance using qPCR (A) and *nosZ* mRNA levels using qRT-PCR (B) in soil incubated over 48 h following addition of glucose at 0 (G0) (□) or 500 mg C-glucose /kg dry soil (G500) (■). Standard curve descriptors of quantification of *nosZ* gene numbers and detection levels: y = -3.35x+ 43.0, R^2 = 0.990, E = 99.0%, no template control (NTC) = undetected. Standard curve descriptors of quantification of *nosZ* transcripts and detection levels: y= -3.38x + 38.4, R^2 = 0.994, E = 97.4%, NTC = undetected. Values are means ± SEM (*n*=4).

Effect of glucose on analytical measurements

Glucose addition resulted in a significant (p = 0.048) decrease in soil NO_3^- concentration, with average values of 425 and 373 mg NO_3^--N /kg dry soil in the G0 and G500 treatments, respectively (Figure 1A). The EOC concentration significantly ($p < 0.001$) increased following glucose addition with average values of 37 and 367 mg C /kg dry soil in the G0 and G500 treatments, respectively (Figure 1B). Reduction in EOC concentration over time indicated that glucose was metabolized.

Over the 48h incubation period, there was no significant difference between cumulative N_2O production from soil incubated without C_2H_2 (i.e. N_2O emissions) or with C_2H_2 (i.e. total denitrification) (Figure 2A), indicating that gaseous emissions from denitrification occurred primarily as N_2O. Cumulative denitrification was significantly ($p = 0.002$) increased by glucose addition (Figure 2A). Addition of glucose significantly increased respiration (cumulative CO_2 emissions) (Figure 2B). Similarly, soil amendment with glucose and other sources of organic carbon such as plant residues or manure have previously been shown to increase denitrification activity, and the increase in denitrification was commonly related to the increase in respiration (Dandie *et al.* 2007; deCatanzaro *et al.* 1985; Miller *et al.* 2008; Miller *et al.* 2009).

Molecular analysis of denitrifier abundance and mRNA levels

Despite the increases in respiration and denitrification in response to glucose addition, there were no measurable changes in the $nirS_p$ (Figure 3A) and *nosZ* (Figure 4A) abundance in soil over the 48 h incubation period. Previous studies also found that the *nosZ*-bearing denitrifier community did not increase in abundance after the addition of glucose to anoxic soil microcosms in 6 day incubations (Miller *et al.* 2008). In contrast, glucose addition at 250 mg-C /kg dry soil increased abundance of *P. mandelii* and closely related species quantified using *cnorB* primers ($cnorB_p$) in anoxic soil microcosms (Dandie *et al.* 2007, Miller *et al.* 2008). In soil amended with glucose, $nirS_p$ gene transcript abundance was increased compared with unamended soil only at 4 h ($p = 0.009$) (Figure 3B). The increase in $nirS_p$ transcripts occurred without a measurable increase in $nirS_p$ abundance, suggesting this increase was through increased mRNA levels per cell. Surprisingly, *nosZ* mRNA levels were not significantly affected by glucose addition and did not change significantly over time during the 48 h incubation ($p = 0.320$) (Figure 4B). Glucose is commonly thought to induce denitrification gene expression through oxygen depletion resulting from increased microbial respiration, however in this experiment where soil anoxic conditions were implemented by changing the headspace gas composition, such a response would not occur.

We hypothesized that the measured denitrification increase in glucose amended soil was due to an increase in denitrifier abundance and/or denitrification gene mRNA levels. Soil amendment with glucose increased microbial respiration and denitrification without a significant increase in abundance of *nosZ* and $nirS_p$ denitrifier communities and with a measurable and transient increase in transcripts for $nirS_p$ only. Therefore, under the experimental conditions used, the increase in denitrification activity was not well linked to denitrifier gene copy and mRNA suggesting that enzyme activity might be important in understanding the control of N_2O emissions in soil systems.

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Molecular characterization of soil fungal communities in paddy soils

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Abstract
Soil fungal communities are important for soil ecosystem functioning. In particular, they are the most important group of organisms involved in decomposing organic matter, especially for resistant compounds such as lignin. Therefore, fungi have an important impact on the decomposition of straw in soil.
In paddy soils, fungal communities are predominant only after drainage and during post-harvest fallow conditions as they need oxygen which is limited under flooded conditions. To investigate the influence of soil management practices on fungi in paddy soils, the period of drainage after flooding was simulated in soil microcosms. In addition, different rice straw applications were tested in an incubation experiment. Molecular analysis including DNA-extraction and PCR/DGGE-analysis was used to characterize the soil fungal communities in these experiments. The resulting dendrograms show clear shifts and correlations between the simulated water stages and rice straw applications.

Key Words
Soil fungal communities, molecular microbiology, DGGE, 18S rDNA, paddy soil.

Introduction

Knowledge about fungi in paddy soils is less compared to other environments, although fungi might play an important role in the decomposition of rice straw, which is up to now almost completely burned. The abundance of fungi in flooded paddy soils is much lower than in upland soils (Kyuma 2004). In general, microorganisms in paddy soils are affected by rapid changes of their habitats caused by the changes between flooding and drainage (Lennartz *et al*. 2009).

There is a great need in soil science to develop novel methods allowing a detailed description of soil fungal diversity without cultivation to improve the understanding of soil fungi and their ecology. Therefore, nucleic acid extraction approaches have become more widely applied in investigations of soil fungi in recent years (van Elsas *et al*. 2000). Direct DNA extraction from soil, coupled with polymerase chain reaction amplification and community profiling techniques, has been proved successful in investigations of bacterial ecology and promises progress for describing the taxonomic and functional characteristics of soil fungal communities (Anderson and Cairney 2004).

The aim of this study is to apply techniques of molecular microbiology to analyse shifts in soil fungal communities in paddy soils. Therefore, two experiments will be established, simulating (i) the period of drainage during cultivation of rice on differently-textured paddy soils and (ii) different rice straw applications on a clay-rich paddy soil.

Methods

Microcosm experiment
A microcosm experiment was set up in a climate chamber in order to simulate a total vegetation period of rice under controlled conditions. Therefore, undisturbed soil microcosms (25 cm in diameter, 30 cm height) were taken at three selected sites in China (Table 1). Soil sampling for the molecular characterization during soil drainage was performed at 111 days after transplanting (DAT) and 118 DAT. These dates are representing the last day under flooded conditions and the day after one week of drainage, respectively. Soil samples were stored at -20 °C until further processing.

Incubation experiment
In order to investigate the microbial response of different rice straw applications in paddy soil an incubation experiment was set up. Therefore, 50 g of homogenized soil (HC) was mixed with shredded rice straw (S) or burned rice straw (bS) and incubated in 250 ml bottles at 25 °C for seven weeks (Table 2). Soil samples were taken after one day and 44 days of incubation for all simulated treatments.

Table 1. Soil description.

Soil	Location	Cultivation	Parent material	Classification (WRB)	Sand (%)	Silt	Clay	Texture (USDA)
LC	Liu Jia, Yujiang County, Jiangxi Province, PR China	Flooded rice - rice rotation	Red Sandstone	Hydragric Anthrosol	61.3	26.2	12.5	Sandy loam
MC	Sun Jia, Yujiang County, Jiangxi Province, PR China	Flooded rice - rice rotation	Quaternary Red Clay	Anthraquic Cambisol	32.7	44.0	23.3	Loam
HC	Jinjiaba, Wujiang County, Jiangsu Province, PR China	Rape - flooded rice rotation	Alluvial Clay	Stagnic Anthrosol	0.9	58.1	41.0	Silty clay

Table 2. Incubation experiment setup.

Treatment	Description	Rice straw (g/kg)
0S	without rice straw	0
bS	burned rice straw *	8.5*
1× S	1× rice straw	8.5
2× S	2× rice straw	17.0

* amount of 1× rice straw before burning; FC: field capacity

Characterization of soil fungi using DGGE

Extraction and purification of DNA from soil samples was carried out using the FastDNA SPIN Kit for Soil (Q-BIOgene) with a modified protocol. The procedures of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) were modified according to Vainilo and Hantula (2000) using 18S rRNA-based primer pairs. DGGE-gels were documented and dominant bands were selected for subsequent sequencing. Dendrograms were constructed from the distance similarity matrix computed by the Nei-Li option and followed by the neighbour joining tree-construction method (Hampl *et al*. 2001; Nei and Li 1979).

Results

The amount of extracted soil fungal DNA varied between the investigated soils according to their clay content (LC>MC>>HC). The primer combination NS1f/FR1 (Pennanen *et al*. 2001) was suitable to amplify 18S rDNA regions. The PCR-products of both experiments were used for the DGGE-analysis. The resulting DGGE-gel (Figure 1a) shows differences in the pattern and intensities of bands of all soils, treatments, and irrigation stages representing shifts in the soil fungal community. The dendrogram of the gel-analysis shows a close relation of the soils LC and MC (Figure 1b). In both soils the fungal diversity between the wet and dry stage differs within a cluster. The HC soil of the microcosm experiment forms a cluster with the treatments without or burned rice straw application in the incubation experiment. The rice straw applications (1× and 2×) are representing an own cluster which is separated from all other samples in the dendrogram. In the incubation experiment, both investigated incubation times have an influence on the soil fungal diversity as they form own clusters for the treatments with rice straw (1× and 2×) and without rice straw (0 and burned), respectively.

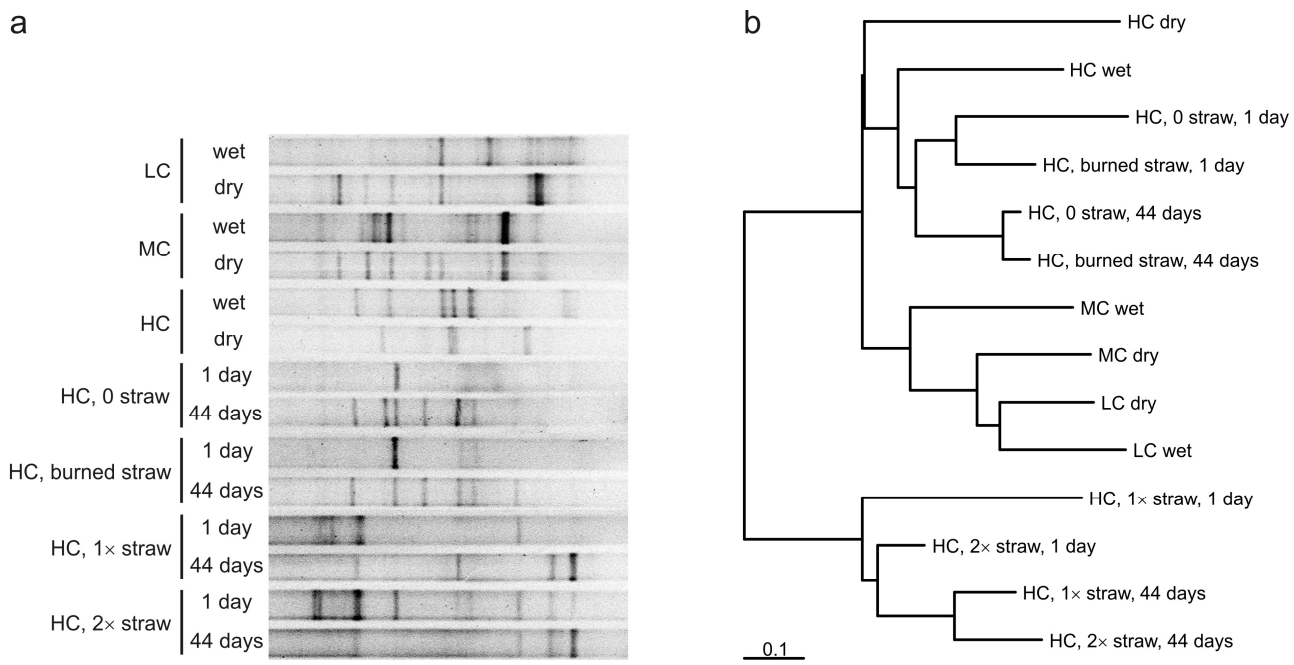

Figure 1. 18S rRNA-based analysis of fungi in the experiments. (a) DGGE gel; (b) dendrogram of the DGGE-analysis.

Conclusion

The results of the DNA-based DGGE-analysis showed shifts of soil fungal communities in both experiments. Within the microcosm experiment the discrimination of the differently-textured soils was clear. The differences between the HC soil and the soils with lower clay content (LC and MC) were very distinct when DGGE-analysis was applied. In the incubation experiment, the differences between the treatments with rice straw (1× and 2×) and the treatments without or burned straw were very clear after DGGE-analysis. Within these treatments, the incubation time had a noticeable influence on the soil fungal community.

The simulated water stages in the microcosm experiment had an impact on the shift of the soil fungal community during the period of drainage. As it has been reported by Kyuma (2004), flooded paddy soils contain less fungi compared to upland soils. As fungi are the most important microorganisms for the decomposition of straw (Finlay 2007), there is more research needed on the occurrence of soil fungi under flooded conditions and after incorporation of rice straw.

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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

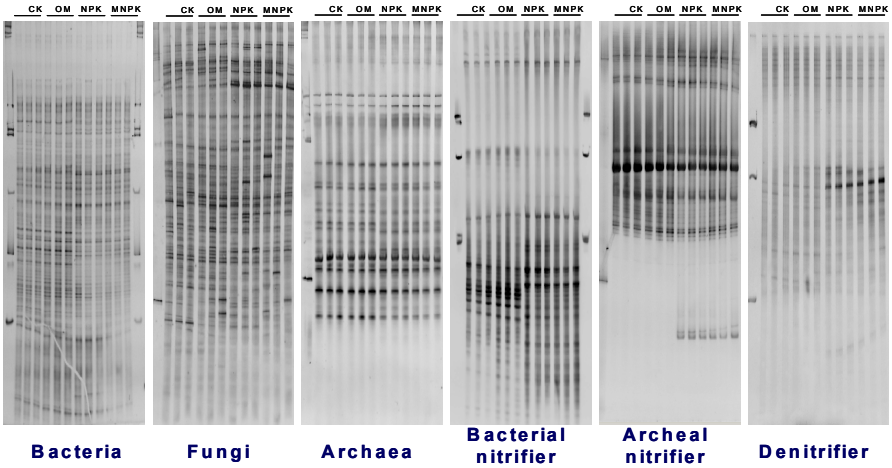

Figure 1. The DGGE profiles of bacteria, fungi, archaea, bacterial and archaeal nitrifiers, denitrifier with neither mineral fertilizer nor organic manure (CK), mineral fertilizer (NPK), organic manure (OM) and both mineral fertilizer and organic manure (MNPK). Each treatment has 3 replications.

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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Taxon-specific responses of soil bacteria to the addition of trace or low amounts of glucose

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Abstract
When small or trace amounts of labile C substrates enter the soils either a priming effect or a triggering effect is induced but the identities of the microorganisms involved remains largely unknown. Here, we modified the Stable Isotope Probing (SIP) protocol to accommodate low level carbon pulses, such that their fate within the microbial community could be determined. Soils were amended with different concentrations of [^{14}C] glucose (150, 50 and 15 and 0 µg C /g soil) prior to rRNA extraction and equilibrium density centrifugation. Subsequent denaturing gradient gel electrophoresis (DGGE) analysis, sequencing of bands and multivariate statistics was then used to identify the taxon specific responses at different glucose concentrations. [^{14}C] RNA enrichment was restricted to *Bacillus, Pseudomonas, Burkholderia* and β-proteobacteria and the concentration of glucose determined the magnitude of the community response. *Pseudomonas* was only enriched at 150 µg C /g soil whilst *Bacillus* and *Burkholderia* although present at 150 µg C /g soil, were more abundant at trace amounts (15-50 µg C /g soil). Glucose amendment also impacted indirectly on the community changing the [^{12}C] RNA signal of *Bradyrhizobium,* Actinobacteria *Nitrosomonas* and Acidobacteria which probably reflects changes in substrate availability and microbial interactions.

Keywords
RNA radioactive isotope probing (RNA-RIP), [^{14}C] glucose, Bacterial Community Structure, Priming Effect, Triggering Effect, Soil organic matter (SOM) turnover.

Introduction

A significant portion of degradable carbon entering agricultural soils from plants is in the form of glucose which is readily degraded by microorganisms. Soil organic matter (SOM) represents a major carbon sink in the biosphere (Fontaine *et al.* 2003) but its turnover is often limited by the availability of labile C substrates, such as glucose, that are required for growth, maintenance and the production of extracellular enzymes (Schneckenberger *et al.* 2008) in most soils. Although, additions of these substrates to soil has been shown to increase microbial activity, accelerate SOM turnover and induce a priming effect, the high application rates (1000-5000 µg C /g) used in earlier studies (e.g. Wu *et al.* 1993) does not reflect the natural C concentrations in soil since inputs can be small and infrequent (Schneckenberger *et al.* 2008). Therefore, recent studies have sought to amend soil with substrates at concentrations more akin to those found in soils (De Nobili *et al.* 2001; Schneckenberger *et al.* 2008) but there is still much debate over whether this induces a priming effect or triggering effect (De Nobili *et al.* 2001) where CO_2 evolution is derived from endocellular reserves. Furthermore, the mechanisms and the identities of the microorganisms involved remain largely unresolved and better methodologies are required.
Stable Isotope Probing (SIP) approaches offer the possibility of linking microbial identity with metabolic function by coupling specific biogeochemical tracers to highly resolved phylogenetic markers, such as the 16S rRNA gene (Whiteley *et al.* 2006). However, the relatively high level of substrate used to label nucleic acids prior to density separation (Whiteley *et al.* 2006) has rendered it unsuitable for soil priming studies so far. The aim of the work reported here was to modify the RNA-SIP procedure to accommodate low level carbon pulses within the range 15–150 µg C /g that are more in line with available C concentrations in soil. To this end, soils were amended with [^{14}C] glucose prior to nucleic acid extraction and equilibrium density centrifugation ([^{14}C] RIP, radioisotope probing). Confirmation that RNA-SIP could discriminate between 'heavy' [^{14}C] RNA and 'light' [^{12}C] RNA at these low C levels was verified by autoradiography, visualised and quantified. Subsequent DGGE analysis of the fractions and subsequent multivariate statistics was used to investigate the relationship between bacterial community structure and different glucose concentrations.

Methods

Microcosm set-up and RNA-radioisotope labelling (RIP) procedure

Soils were amended with different concentrations of [^{14}C] glucose (150, 50, 15 μg C /g soil and control [no amendment, 0 μg C /g]) and incubated at 22 ° C for 4 days to ensure adequate enrichment. Following [^{14}C] glucose amendment, nucleic acids were extracted using the phenol-chloroform-CTAB method (Griffiths *et al.* 2000). The 'heavy' [^{14}C] labelled RNA was separated from the 'light' [^{12}C] unlabelled RNA was separated using equilibrium density centrifugation. The existing [^{13}C] RNA-SIP protocol (Whiteley *et al.* 2007) was adapted to accommodate the use of [^{14}C] labelled glucose and then optimised for low C additions. The extracted RNA was added to Caesium trifluoracetate (CsTFA) and spun in an Optima TLX ultracentrifuge at 60,000 rev/min and 20 °C for 37 h to form a density gradient. Two blank reactions were included in every run to provide a reference gradient so that a density profile could be calculated after fractionation. Gradients were fractionated from below by displacement with water by using a Beckman Fraction Recovery System and a syringe pump at a flow rate of 3.3 μl/s. Fractions were collected every 30 s, giving 30 fractions per gradient and the RNA was isolated from each fraction by precipitation with ice-cold isopropanol. The [^{14}C] RNA radioisotope probing (RIP) approach was evaluated and verified by autoradiography to confirm the location of the [^{14}C] fractions.

Molecular community profiling

The RNA from each fraction was subjected to reverse transcription (RT)-PCR and the PCR-products were analysed by denaturing gradient gel electrophoresis (DGGE). Unique bands were excised from the DGGE gels, re-amplified and sequenced. Replicate DGGE gels (x3) were digitized and analysed using Bionumerics gel analysis software to generate a data matrix of relative taxon abundance (band intensity) for each community (lanes). A corresponding data matrix of explanatory variables (glucose concentration, buoyant density) was prepared and canonical correspondence analysis (CCA) was used to explore the relationship between community structure and glucose concentrations.

Results and discussion

The [^{14}C] RNA-RIP approach clearly resolves the 'heavy' fractions ([^{14}C] labelled RNA) from the 'light' fractions ([^{12}C] unlabelled RNA) which was verified using autoradiography. Interestingly, the RNA-RIP has highlighted the existence of [^{14}C] intermediate fractions. A range of buoyant densities could have resulted from variations in the amount of [^{14}C] enrichment between glucose assimilating taxa or differences in G+C content between bacterial taxa. This 'effect' will be accentuated here by the [^{14}C] RNA (compared to [^{13}C] RNA) since the separation distance is greater along the gradient.

As expected, DGGE analysis showed a distinct shift in community structure following the addition of [^{14}C] glucose at all concentrations and was characterised by an increase or decrease in the relative abundance of particular bands (Figure 1). The canonical correspondence analysis (CCA) biplots in Figure 2 showed that the community response in both the 'heavy' and 'light' fractions differed according to the concentration of glucose amendment. For both fractions, the differences in community structure were most pronounced in soils amended with 150 μg C /g soil. In the 'heavy' RNA fractions there was a clear separation between soils amended with 150 μg C /g soil and those receiving 50 and 15 μg C /g soil; the community structures in the latter were more similar to each other. This suggests that at low or trace C inputs (so-called trigger molecule concentrations) the microbial community response is quite distinct from that seen at higher concentrations. A possible explanation is that there is insufficient energy to activate the majority of the community at this level of amendment (Wu 1993; De Nobili *et al.* 2001) and the energy is used for internal microbial metabolism instead possibly inducing a 'triggering effect' (De Nobili *et al.* 2001).

To investigate these differences further, bands that changed in relative intensity following amendment were excised from the DGGE gels, reamplified and sequenced. [^{14}C] RNA enrichment was restricted to *Bacillus, Pseudomonas, Burkholderia* and β-proteobacteria. This is not unexpected since competition for a limited resource typically results in the dominance of one or a few populations with the highest growth rates (Fontaine *et al*. 2003). Also, these bacterial groups are particularly adept at responding to a variety of labile C compounds entering soils across a range of different ecosystems and have been described as r-strategists or (Bernard *et al.* 2007). *Pseudomonas* was only enriched at 150 μg C /g soil which is consistent with their putative roles as r-strategists whilst *Bacillus* and *Burkholderia* although present at 150 μg C /g soil, were most prevalent at the lower glucose concentrations (15-50 μg C /g soil) which could mean they have evolved survival strategies to remain 'metabolically alert' during periods of starvation (De Nobili *et al.* 2001). Glucose amendment also impacted indirectly on the community by changing the abundance of taxa in the 'light' fractions and this probably reflects changes in substrate availability and microbial interactions. The

increase in the abundance of Actinobacteria and *Bradyrhizobium* in the 'light' fractions has been previously reported following substrate amendment and was linked to enhanced SOM degradation and priming (Bernard *et al.* 2007). According to Fontaine *et al.* (2003) the fast-growing r-strategist that initially utilise the substrate will be superseded by slow-growing K-strategists able to decompose recalcitrant organic compounds (Fontaine *et al.* 2003). Although this was not proven here, *Bradyrhizobia* are oligotrophic slow-growing bacteria whilst Actinobacteria are known for their ability to degrade recalcitrant material. There was a marked decrease in the relative abundance of Acidobacteria and α-proteobacteria (*Nitrosomonas*) and this has been observed before (Bernard *et al.* 2007). Explanations include the possibility that glucose may have induced changes in the soil microenvironment and community structure due to enhanced heterotrophic activity leading to reduced oxygen availability and a community dominated by facultative and anaerobic microorganism (Griffiths *et al.* 1999). Also, with accelerated microbial growth there is higher potential for antagonism through either competition for resources or by the secretion of antimicrobial metabolites (Griffiths *et al.* 1999). Finally, these oligotrophs could be sensitive to high osmotic stress (Griffiths *et al.* 1999) and their uptake mechanisms may have become swamped at the higher glucose concentrations.

Conclusion

This study demonstrates that the response of soil bacteria following amendments with low level pulses of glucose is restricted to a few specific taxa affiliated with *Bacillus, Pseudomonas*, *Burkholderia* and *β*-proteobacteria. The amount of glucose added to soil determines the magnitude of the bacterial response. Trace or small glucose amounts were accompanied by an increase relative abundance of *Bacillus* and *Burkholderia* whilst at higher glucose levels *Pseudomonas* dominated the community. This is probably due to more energy being available for growth. Glucose amendment also impacted indirectly on the community by increasing the abundance of *Bradyrhizobium* and Actinobacteria whilst reducing the abundance of *Nitrosomonas* and Acidobacteria in the 'light' fractions. This probably reflects changes in substrate availability and microbial interactions. Overall, these results show that RNA-RIP can be used successfully to study the fate of labile C substrates, such as glucose in soil.

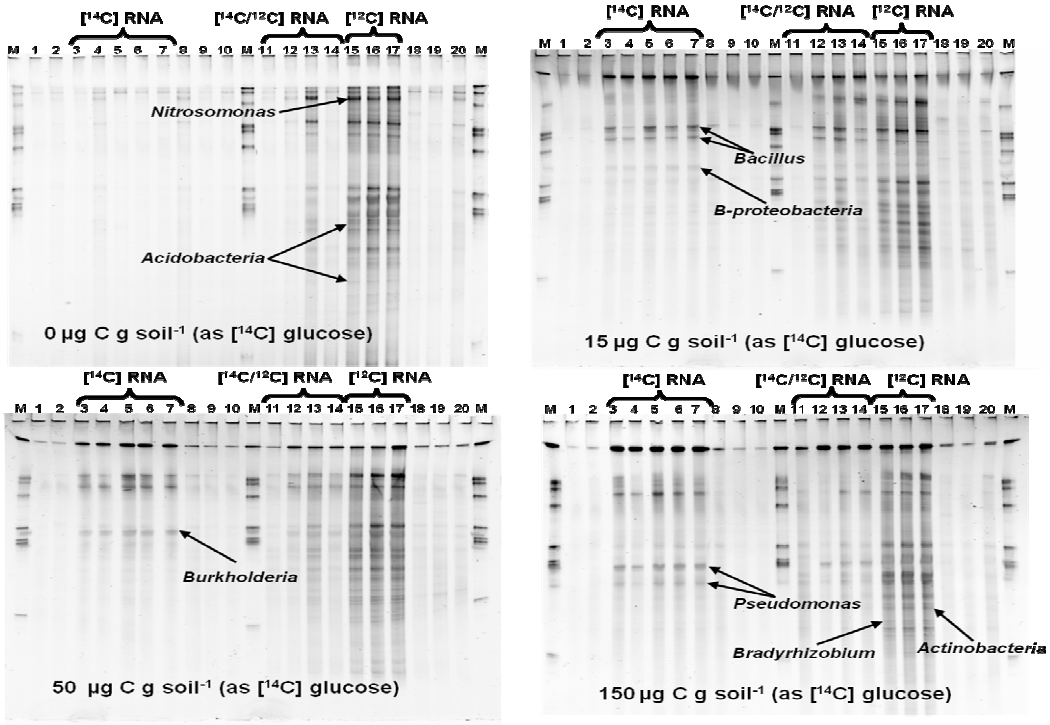

Figure 1. RT-PCR-DGGEgels comparing the bacterial community profiles recovered from first 20 fractions along the RNA-SIP density gradient after 36 h of centrifugation. RNA was extracted from soil in microcosms amended with different concentrations of [^{14}C] glucose (0, 15, 50, and 150 µg C /g soil) after 4 days of incubation. The 'heavy' fractions (3-7) containing the [^{14}C] labelled RNA, the 'light' fractions (15-17) containing the [^{12}C] unlabelled RNA and the 'intermediate' fractions (11-14) containing [^{14}C/^{12}C] labelled RNA. M indicates the marker lane and numbers represent fraction number. Arrows indicate the bands that were excised from the gel, reamplified and sequenced.

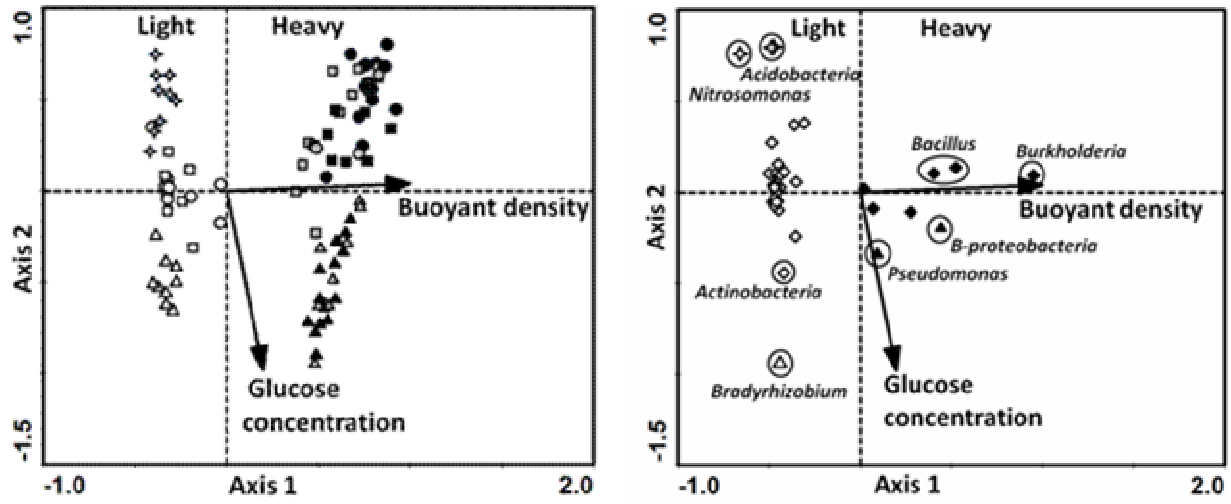

Figure 2a) Biplot from canonical correspondence analysis (CCA) showing the change in bacterial community composition (as judged by DGGE banding patterns) along the buoyant density gradient and with increasing [^{14}C] glucose concentration [first two axes account for 48% of total variance]. Points on the graph represent the bacterial communities recovered from 'heavy' fractions (3-7) containing [^{14}C] labelled RNA (Black■), 'intermediate' fractions (11-14) containing [^{14}C/^{12}C] labelled RNA (Grey ■) and 'light' fractions (15-17) containing [^{12}C] unlabelled RNA (White □) over the first 20 RNA-SIP fractions. The different concentrations of [^{14}C] glucose 0 (star ✦), 15 (circle ●), 50 (square ■) and 150 (triangle ▲) µg C/g soil were assigned accordingly. Arrows represent the explanatory variables [Buoyant density and glucose enrichment concentration] and the direction of the arrow indicates increasing density or concentration

2b) Biplot shows the distribution of bacterial taxa along the same density buoyant gradient. Points represent the bacterial taxa recovered from 'heavy' fractions (3-7) containing [^{14}C] labelled RNA (Black◆) and 'light' fractions (15-17) containing [^{12}C] unlabelled RNA (White ◇). The taxa were assigned to groups based on their abundance at different glucose concentration: taxa only found in the control samples (✦), taxa found in all [^{14}C] glucose concentration amendments (◇/◆) and taxa more abundant in 150 µg C /g soil amendment (△/▲).

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Utilising microarray technology to investigate soil microbial ecology

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Abstract
Microarray technology provides a tool for assessing the transcriptional response or abundance of thousands of microbial functional or structural genes concurrently. Two new microarrays have been designed to detect a range of microbial functional gene targets for enzymes associated with the cycling of soil C, N, P and in antibiotic production, biodegradation and methane oxidation. A Phylochip structural array based upon 16S ribosomal RNA sequences of bacteria and archaea was also used to examine soil microbial communities. The three microarrays have been used in two experimental designs, one based on contrasting soil types and land-uses, the second in a climate change scenario of elevated CO_2 and warming. The functional microarrays provided valuable tools for the quantitative description of key soil microbial process associated with soil health, that are essential for the productive and sustainable management of natural and agricultural ecosystems. The Phylochip structural microarray elucidated major differences in bacterial community structure based upon the effects of climate change. Collectively, microarray technology represents a significant improvement in rapid and precise measurements of soil functions and responses to management. It therefore has enormous potential for application in land management decision support processes.

Key Words
Microarray, microbial ecology, functional genes, 16S, climate change

Introduction
Australian soils are under increasing pressure to provide a range of agroecological goods and services to meet the growing global demand for food, fibre and bioenergy. Current land-use practices are likely to influence soil ecosystem health and the underlying microbially-mediated functions related to plant nutrient supply (Kibblewhite *et al.* 2008). Furthermore, the influence of climate change on soil ecosystem health related to plant-available soil moisture and uptake efficiencies of soil-immobile nutrients will require detailed analyses of below ground process. A combination of high resolution molecular tools as well as lower resolution methods such as microbial biomass, gross N mineralization, microbial immobilization and net N mineralization may assist in predicting how microbial activity and rates of soil carbon and nitrogen cycling will change with increases in atmospheric CO_2.

Microarrays offer specific, sensitive, quantitative, and high-throughput tools for microbial detection, identification, and characterisation in soil ecosystems (Blalock 2004). While there are issues involved with their adoption such as the cost of the technology, sample preparation for hybridisation and the data analysis of information captured from thousands of probes simultaneously, progress is being made in the use of microarray technology for studying microbial ecology. Phylogenetic microarrays based upon the conserved marker 16S ribosomal RNA (rRNA) gene have been used to compare the relatedness of communities in different environments (Brodie *et al.* 2006). Functional gene arrays based upon functional genes that code for proteins catalysing processes such as the carbon, nitrogen, and sulfur cycles have been utilised to ascertain the potential of microbial populations to conduct these processes in soil (Wu *et al.* 2001).

Here we report on the development and demonstration as a proof of concept of two microarrays designed using CombiMatrix CustomArray technology to assess microbial functions associated with the health of Australian soils and climate change. The Soil Health Array was designed to quantify the microbial activity associated with environmental functions including nitrogen and phosphorus cycling, organic compound degradation, and antibiotic and plant growth promoting substances in a range of contrasting soils, in order to tentatively identify the environmental factors driving the change in soil function. The functional nitrogen and carbon (FuNC) array was designed to focus on nitrogen and carbon cycling by microbes in an environment

under the simulated effects of climate change with elevated CO_2 and warming at TasFACE (a Free Air Carbon dioxide Enrichment facility) in Tasmania. The Phylochip structural array, developed at the Lawrence Berkeley National Laboratory, was also used in parallel with the FuNC array to investigate the effects of climate change on microbial community structure at TasFACE. The use of microarrays will provide valuable tools for the quantitative description of key soil microbial processes and microbial community structure associated with climate change and soil health. Knowledge of soil microbial communities and their function gained from the use of arrays can be used to inform decision making for the productive and sustainable management of natural and agricultural ecosystems.

Methods

Soil Ecosystems

As a proof of concept for the Soil Health Array, soil samples were collected from paired sites of two different land-uses (managed and remnant) within two different geomorphic zones and Soil Orders in Victoria. Three locations were sampled in the 'Eastern Uplands' of North-East Victoria where the Dermosol soil is characterised by a strongly acidic surface soil and three locations in the 'North Western Dunefields and Plains' zone of North-West Victoria where the Calcarosol soil has a neutral to slightly alkaline surface soil. The 'managed' sites were agricultural sites that were cropped or under pasture, with varying levels of agricultural inputs. The 'remnant' sites comprised small parcels of land that were either State Parks or shelter belts where native plant ecosystems remained. At each location, soil cores were collected from the paired land-use sites, and DNA extracted using a Bio101 Fast DNA Spin Kit. The sites studied were chosen because of the additional metadata (chemical and microbial) available for comparison to the microarray gene data.

The FuNC array and Phylochip were utilised to examine the effects of climate change on the soil microbial community. TasFACE in Tasmania, Australia, is an experimental field site for examining the effects of increased temperature and atmospheric carbon dioxide concentrations on a native pasture. TasFACE has been running for more than 8 years with changes in the plant community documented over time (Hovenden *et al.* 2008). Soil samples were collected in October 2007 (the 6th year of treatment). Sixteen different treatments were tested to examine interactions between elevated CO_2 (FACE or control), elevated temperature (warmed or unwarmed), plants (the dominant grass *Themeda* spp. or non-*Themeda*), and soil depth (0-5cm or 5-10cm). DNA was isolated from soil using a MoBio Powersoil kit.

Soil Health Array

Several functional families were targeted for sequence selection including nitrogen cycling, phosphate cycling, 1-carbon oxidation, biodegradation and global activators. Gene sequences from the bacterial and archaeal domains were obtained for 68 functional groups in the nine functional families. Probes were designed from selected sequences using Oligoarray 2.1, with 2933 probes selected. The microarray probes were synthesised on a CombiMatrix 12K CustomArray and replicated four times across the array. Twelve samples of soil DNA (3 μg) were labelled and hybridised to the array and the resultant data analysed to select probes that showed substantial difference across the samples.

FuNC array

Functional families representing the processes of nitrogen and carbon cycling in bacteria, archaea and fungi were targeted for sequence selection for probe design. Carbon cycling functions such as photosynthesis, methanogenesis, C decomposition and, carbohydrate, pectin, cellulose, hemicellulose and lipid catabolism, were represented by 6678 sequences from Genbank. A suite of genes representing N cycle functions such as ammonia and nitrite oxidation, nitrogen fixation, and the reduction of nitrite, nitrate, nitric oxide and nitrous oxide were represented by 20738 sequences. The array included probes that were designed to provide either high levels of coverage within functional groups ("4-hitters") or high levels of specificity to target sequences ("specific"). The microarray probes were designed for a 40K block and synthesised onto a CombiMatrix 90K CustomArray as two replicated blocks. Forty-two samples of soil DNA (1 μg) from TasFACE were hybridised to the array and the resultant data analysed to select probes that showed a substantial difference across treatments.

Phylochip

The Phylochip is a structural Affymetrix array for identifying bacterial and archaeal populations to subfamily level and was designed at LBNL (Brodie *et al.* 2006). The array contains ~300,000 probes that detect 8741 different bacteria and archaea representing two domains, 63 phyla, 136 classes, 262 orders and 455 families.

Forty-two samples of soil DNA from TasFACE were amplified with primers specific to the 16S rRNA subunit of bacteria and the 16S rRNA subunit of archaea, then the resultant PCR products were hybridised to the Phylochip. Fluorescence readings for each array were normalised and data analysed to select probes that showed a substantial difference across treatments.

Results

Soil Health Array

Probes designed for the array mapped to 5456 targets from an input sequence set of 6420 sequences, representing a probe design success rate of 85% for all sequences submitted. The results of the two-way hierarchical clustering of probe signal intensity from the 12 samples for a filtered set of 2770 probes are presented in Figure 1. Hierarchical clustering revealed six distinct probe signal intensity profiles (probe clusters) from across the samples analysed (horizontal clusters on the heat map), while the 12 soil DNA samples demonstrated three major patterns of probe signal intensity (sample clusters) shown as vertical clusters on the heat map. Samples can be seen to cluster on the basis of geomorphic region (eg North-West [NW] or north-East [NE]) or land-use (managed [M] or remnant [R]). Different N cycle genes were present in the probe clusters: ammonia monooxygenase (*amoA*) in clusters 1, 2 and 4; nitrogenase (*nifH*) was dominant in probe clusters 3, 5 and 6; methanol dehydrogenase (*mxaF)* was dominant in clusters 5 and 6; and nitric-oxide reductase (*NOR)* was most abundant in cluster 3. Particulate methane monooxygenase (*pmoA*) was also dominant in probe clusters 1 and 2.

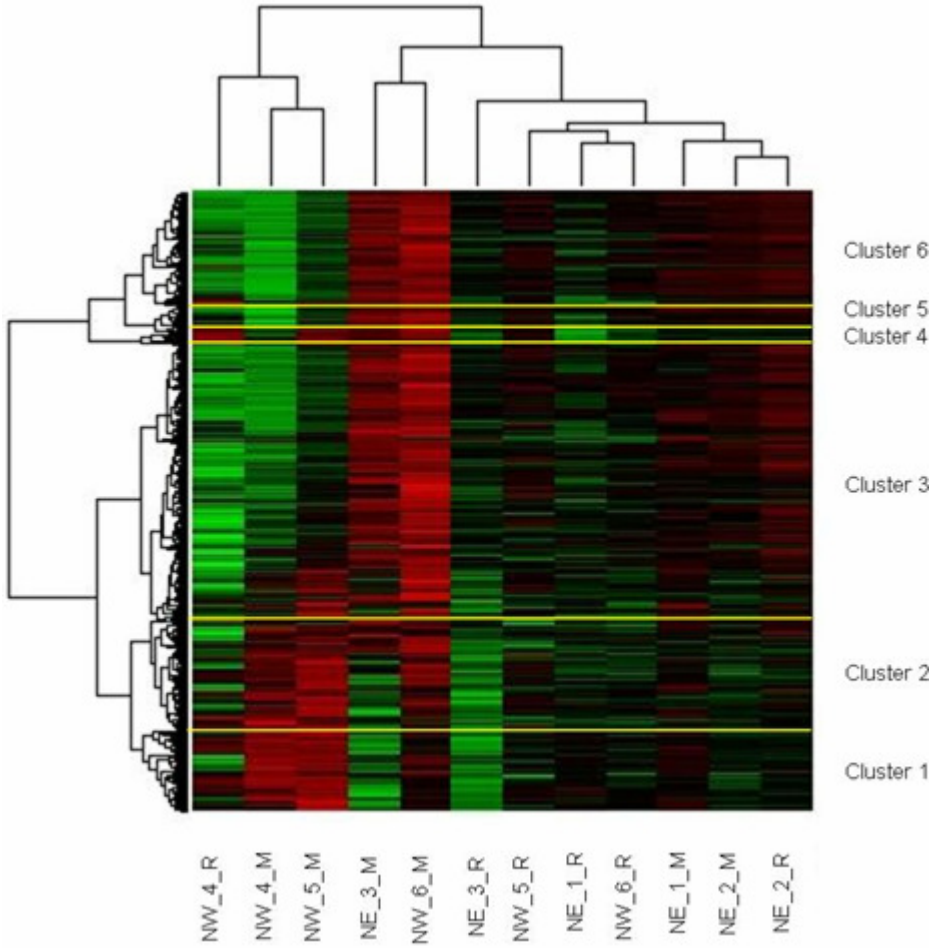

Figure 1. A heatmap generated using hierarchical clustering showing simultaneous relationships between probe signal intensities across and within soil samples. Red coloration indicates an increase in probe signal intensity while green indicates a decrease. Yellow horizontal lines indicate the boundaries between clusters of probes that show similar profiles across the samples.

FUNC Array

Data was collected based on analysis of variance from 7795 probes from functional genes showing strong signals across samples (Table 1). Carbon and nitrogen cycling genes were detected for all factor and interaction terms in the TasFACE experiment, with probe responses being similar between the "4-hitter" and

"specific" probes. Probe signal intensity and the number of probes (proportional representation) from each functional group were summarised for the carbon and nitrogen cycling genes found to have a significant ($P < 0.05$) response. Strong effects on functional gene response were observed for warming (and soil moisture) and FACE (elevated CO_2) treatments, and their interaction with plant communities (C4 species *Themeda* vs non-*Themeda* C3 species).

Table 1. Summary of the number of FuNC array probes significant ($P < 0.05$) within each factor and interaction in a 4 way ANOVA of TasFACE data.

Factor or interaction term	Number of significant probes
FACE (Elevated CO_2)	305
Warming	620
Themeda	23
Depth	28
FACE x Warming	124
FACE x Themeda	241
FACE x Depth	3
Warming x Themeda	168
Warming x Depth	814
Themeda x Depth	2
FACE x Warming x Themeda	611
Warming x Themeda x Depth	4
Warming x Themeda x Depth	31
FACE x Depth x Themeda	9

Phylochip

The Phylochip microarray identified that different phyla and classes were responding to the treatments (factors) and interactions tested at TasFACE. Dominant groups that responded to the different factors were the classes Acidobacteria and Alphaproteobacteria for temperature; Firmicutes and Bacteroidetes for FACE; and Actinobacteria and Bacteriodetes for the grass species *Themeda*. Taxa across all Phyla responded to depth with a decrease in probe fluorescence intensity. Of the interactions tested FACE x Temperature affected the most probes ($n = 103$) with four different phyla, including the classes Actinobacteria, Sphingobacteria, Clostridia, Alphaproteobacteria, Deltaproteobacteria and Gammaproteobacteria responding. Four clusters based on similar probe responses were identified for the FACE x Temperature interaction.

Conclusion

The use of three different microarrays (Soil Health Array, FuNC array and Phylochip) on soil microbial communities has been described. The application of microarray technology to differing soil ecosystems has provided insight to microbial functional processes and community structure for many hundreds of genes simultaneously, and how these may be impacted by Soil Order, land-use or climate change.

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