The effects of stubble retention and nitrogen application on soil microbial community structure and functional gene abundance under irrigated maize

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Abstract

The effects of agronomic management practices on the soil microbial community were investigated in a maize production system in New South Wales, Australia. The site has been intensively studied to measure the impact of stubble management and N-fertilizer application on greenhouse gas emissions (CO_2 and N_2O), N-cycling, pathology, soil structure and yield. As all of these endpoints can be regulated by microbial processes, the microbiology of the system was examined. Soil samples were taken after a winter fallow period and the diversity of the bacterial and fungal communities was measured using PCR-denaturing gradient gel electrophoresis. Stubble and N shifted the structure of bacterial and fungal communities with the primary driver being stubble addition on the fungal community structure ($P < 0.05$ for all effects). Changes in C, N (total and NO_3), K and Na, were correlated ($P < 0.05$) with variation in the microbial community structure. Quantitative PCR showed that *nif*H (nitrogen fixation) and *nap*A (denitrification) gene abundance increased upon stubble retention, whereas *amo*A gene numbers were increased by N addition. These results showed that the management of both stubble and N have significant and long-term impacts on the size and structure of the soil microbial community at phylogenetic and functional levels.

Introduction

The size and structure of soil-borne microbial communities are intimately linked with physicochemical soil properties (Garbeva *et al.*, 2004; Balser & Firestone, 2005) and soil microbial communities sustain many vital ecosystem processes, such as nutrient cycling, decomposition of organic matter and waste, nutrient availability, degradation of pesticides and contaminants, soil structure, and plant growth and health (van Elsas *et al.*, 1997). Assessing the effect of soil management practices on microbial community structure, diversity and activity in soil is therefore critical to advancing the understanding of the functionality, stability, and resilience of managed and natural ecosystems (Kennedy & Smith, 1995).

The ability to manage inputs to soils in agricultural systems provides an excellent opportunity to investigate many areas of microbial ecology in a directed approach. The information provided may ultimately be used to manage agroecosystems towards production, sustainability and environmental targets. Increasingly, ecological investigations assessing microbial community structure and diversity employ culture-independent techniques such as rRNA gene-based PCR-denaturing gradient gel electrophoresis (PCR-DGGE) profiling (Muyzer & Smalla, 1998). Culture-dependent methods are, in general terms, predisposed towards selection and assessment of easily cultured and fast growing microorganisms. Such methods can therefore exert strong experimental bias on r-strategy community assessment, which is not acceptable in broad ecological studies. As such, the use of molecular methods for microbial community analysis has been applied to a wide range of areas in soil microbial ecology.

An irrigated maize monoculture field trial site was established at Whitton, New South Wales, Australia, in 1999 and ran until 2005. The trial was designed to investigate the effect of a range of conservation tillage and N management treatments on maize production, plant health

and soil nutrient cycling (C and N), and to investigate environmental end-points, such as greenhouse gas emission (e.g. N_2O), C-sequestration and soil structural benefits (Harvey *et al.*, 2006; Kirkby *et al.*, 2006; Meyer *et al.*, 2006). Many of these processes are driven by microbial activity, and it is therefore essential to investigate the effect of altering stubble and nitrogen management on the size and structure of the microbial community.

This study examined the effect of stubble and N management on the size and structure of dominant microbial communities in the Whitton field site. Community structure and diversity of bacteria and fungi were assessed by rRNA gene-based PCR-DGGE, and were related to changes in the physicochemical properties of the soil resulting from management treatments. Although rRNA gene-based PCR-DGGE provides information on microbial community structure, it gives little information on functional aspects of these communities. Accordingly, the response to management of key functional groups was measured using real-time PCR. Focus was given towards microbial communities involved in the geochemical cycling of N for the following reasons: (1) N is a key driver for ecosystem fertility but can also act as a pollutant (eutrophication, greenhouse gas); (2) microbial activity controls most N transformations; and (3) methods are available to target conserved genes encoding enzymes involved in N transformations. Amplification of conserved regions of the *amo*A, *nif*H and *nap*A genes was therefore used to assess the relative size of the microbial communities involved in N-fixation, nitrification and denitrification.

Materials and methods

Site, soil collection and processing

The irrigated continuous maize trial site was located in the Murrumbidgee irrigation area, NSW, Australia. Soil was sampled from under four different management practices: stubble retained, with and without N addition; or stubble burnt, with and without N addition. Where stubble was retained, it was rotary-hoed back into the soil at the end of the growing season. Treatments receiving N had fertilizer applied at district practice rates (329 kg N ha^{-1} $year^{-1}$). The N application was split: presowing, 112 kg N ha^{-1} was applied as super phosphate (32% N and 10% P), 5 kg ha^{-1} of liquid N at sowing, 230 kg ha^{-1} urea 1 month after sowing, and a second application of urea 1 week after the first (Kirkby *et al.*, 2006). P was applied across treatments without N addition to balance inputs. Soil from each of the management treatments was collected from four different localities (replicates): two true randomized field blocks and two spatially distant (> 50 m) subreplicates within the blocks. The distance between sampling locations within the field blocks was greater than the distance between blocks. At each sampling point, several small samples of topsoil (1–15 cm) from randomly selected different locations were collected using a hand trowel and mixed into a bulk sample. Soils were sampled in September 2004, which followed the winter fallow period, and dried in a glasshouse to *c.* 3% moisture. Dried soil samples were crushed in a mortar and pestle but were not sieved so as to retain coarse organic matter. The maximum water holding capacity (MWHC) of the soil was determined using Shaw's method (Jenkinson & Powlson, 1976) (MWHC = 58 g water per 100 g soil). The physicochemical properties of soils from the different treatments were determined by the Analytical Services Unit of CSIRO Land and Water, Adelaide. Samples (100 g dry weight) of each of the 16 soils were preincubated at 20 °C for 14 days in the dark at 50% MWHC prior to microbial biomass determination and DNA extraction.

Microbial biomass determinations

Microbial biomass carbon (MBC) was determined using the chloroform fumigation extraction method (Jenkinson & Powlson, 1976). Briefly, for two samples of *c.* 10 g from each of the soils, one sample was directly extracted to determine dissolved C and the second was extracted following chloroform fumigation-lysis of microbial cells (7 days of fumigation). Extractions were performed by adding 30 mL of K_2SO_4 (0.5 M; pH 6.3) to each sample, shaking for 1 h and then filtering through two layers of filter paper (Whatman no. 42) into plastic phials. Total C was determined by infrared detection following combustion at 950 °C on a Formacs series combustion TOC/TN analyser (Skalar Analytical Ltd).

DNA extraction and PCR

From each sample of incubated soil, three 1-g subsamples were processed for DNA extraction using the UltraClean Soil DNA isolation kit (MoBio Laboratories) following the manufacturer's instructions but with mechanical disruption by a FastPrep beadbeater (FP120; Qbiogene). Extracts from subsamples were combined into a final volume of water (150 μL), and DNA quantified using a SpectraMAX Spectrofluorometer with PicoGreen double-stranded DNA quantification reagent (Molecular Probes Inc.).

PCR for bacterial and fungal community analyses was conducted in an Eppendorf mastercycler gradient PCR machine using Hot-Star *Taq* DNA polymerase (1 U per 25 μL) and buffer (Qiagen). Functional gene quantification was conducted on a Stratagene M×3000*P* real-time PCR machine using Qiagen QuantiTect SYBR green 1 mix, with amplification adjusted to background (passive) fluorescence of ROX. C_T values were determined from a software-determined baseline; C_T is the cycle at which PCR

amplification is in exponential amplification above a given threshold. For all genes, PCR amplification was initiated by a hot-start incubation at 95 °C for 15 min, and the amplification cycles were followed by a final elongation step at 72 °C for 5 min. Dissociation curves were conducted at the end of real-time PCR and reaction products were also separated on 1.5% agarose gels to confirm single banding at the expected size. All reactions were conducted in a volume of 25 μL.

Functional gene detection

Functional gene detection for nitrogen fixation was based on PCR of nitrogenase reductase gene (*nif*H) fragments as described by Widmer *et al.* (1999). The nested PCR used primers *nif*H(forA) and *nif*H(rev) for the first-round amplification, and primers *nif*H(forB) (Zehr & McReynolds, 1989) and *nif*H(rev) for the second (Table 1). In addition to the PCR master mix, first-round reactions contained 20 pmol of primers *nif*H(forA) and *nif*H(rev) and 2 μL of DNA (1 : 10 dilution). Second-round amplifications used primers *nif*H(forB) and *nif*H(rev) and 2 μL of 1 : 10 dilution first-round PCR product as template. First-round PCR cycling conditions were: 30 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C 2 min. Second-round PCR cycling conditions were: 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s.

Measurement of genetic potential for denitrification was based on detection of periplasmic nitrate reductase (*nap*A) following Flanagan *et al.* (1999). The first round of the nested PCR used primers V16 and V17, whereas the second round used primers V66 and V67 (Table 1); primers were used at 15 pmol. In the first round of PCR, 2 μL of 1 : 10 dilution DNA was used as template for amplification. In the subsequent round of PCR, 2 μL of the first-round reaction mixture was used as the DNA template. Both first- and second-round PCR followed the same cycle conditions: 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min.

PCR was carried out to amplify a fragment of the ammonia monooxygenase gene (*amo*A), following the method and using the primers described in Stephen *et al.* (1999) as modified from Rotthauwe *et al.* (1997). PCR amplifications contained 10 pmol each of primers *amo*A-1F* and *amo*A-2R (Table 1), 10 μg bovine serum albumin (Roche Diagnostics Ltd), and 2 μL of soil-extracted DNA. PCR conditions consisted of 35 cycles of 92 °C for 1 min, 50 °C for 1 min and 68 °C for 45 s.

Community structure analysis

The structure of the soil bacterial and fungal communities was based on rRNA gene-targeted PCR-DGGE. Bacterial community-specific PCR used universal bacterial primers F968-GC and R1401 as described in Duineveld *et al.* (1998). Each PCR contained primers at 20 pmol and used 2 μL of 1 : 10 diluted DNA extract. The PCR cycle used a touchdown procedure with a reduction in annealing temperature from 67 to 57 °C over the first 20 cycles, and at 57 °C for the 20 cycles thereafter. Each cycle had denaturation at 94 °C for 1 min, annealing for 1 min and extension at 72 °C for 1 min. PCR of fungal internal transcribed spacer (ITS)-RNA genes was conducted with primers ITS1F-GC and ITS2* (Table 1). Each PCR contained 20 pmol of each primer and used 2 μL of 1:10 diluted soil DNA. Thermocycling conditions were 40 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min. Confirmation of successful bacterial and fungal PCR

Table 1. Primers for functional genes and community analysis

Gene	Primer	Sequence (5′ → 3′)	Reference
*nif*H	*nif*H(forA)	GCI WTI TAY GGN AAR GGN GG	Widmer *et al.* (1999)
	*nif*H(forB)	GGI TGT GAY CCN AAV GCN GA	Zehr & McReynolds (1989)
	*nif*H(rev)	GCR TAI ABN GCC ATC ATY TC	
*Amo*A	*amo*A-1F*	GGG GHT TYT ACT GGT GGT	Stephen *et al.* (1999)
	*amo*A-2R	CCC CTC KGS AAA GCC TTC TTC	Rotthauwe *et al.* (1997)
*nap*A	V16	GCN CCN TGY MGN TTY TGY GG	Flanagan *et al.* (1999)
	V17	RTG YTG RTT RAA NCC CAT NGT CCA	
	V66	TAY TTY YTN HSN AAR ATH ATG TAY GG	
	V67	DAT NGG RTG CAT YTC NGC CAT RTT	
16S	F968-GC	GCC CGG GGC GCG CCC CGG GCG GGG CGG GGG CAC GGG GGG AAC GCG AA GAA CCT TAC	Heuer & Smalla (1997)
	R1401	GCG TGT GTA CAA GAC CC	
ITS[A]	ITS1F-GC	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CTT GGT CAT TTA GAG GAA G TAA	
	ITS2*	TTY GCT GYG TTC TTC ATC G	Gardes & Bruns (1993)

Underlined nucleotides denote GC-clamp.

Degenerate bases (IUPAC abbreviations): R, AG; Y, CT; M, AC; W, AT; S, CG; K, T+G; B, CGT; V, A+C+T; D, A+T+G; H, A+T+C; N, ACGT. I, inosine.

*The fungal-specific primer ITS1F was designed by Assistant Professor D. Lee Taylor, University of Alaska, Fairbanks.

was achieved by running 2 μL of PCR product on a 1.5% agarose gel, staining with ethidium bromide and visualizing under UV light. The remaining reaction mix was used for DGGE analysis.

DGGE analysis of amplified bacterial 16S and fungal ITS rRNA genes was performed on the DCode Universal Mutation Detection System (Bio-Rad). The polyacrylamide gels (7% w/v) contained a linear formamide/urea gradient ranging from 45% to 55% (bacterial) or 20% to 50% (fungal) denaturant. Gels were run for 16 h at 60 V and 60 °C, silver-stained (Bio-Rad silver stain kit), dried and scanned (Epson expression 1680 transparency scanner). From the gel images (e.g. Fig. 1), DNA band positions and intensity were detected using TOTALLAB software (Nonlinear Dynamics Ltd). Background signal was removed using the rolling ball method and 43 and 59 different band positions were detected for bacterial 16S rRNA gene and fungal ITS gels, respectively. Each band position was inferred as a distinct operational taxonomic unit (OTU) and relative band intensity was used as an indication of relative abundance (discussed in Fromin *et al.*, 2002).

Statistical analysis

An ecological distance matrix was compiled on fourth root transformed DGGE banding data (to reduce overweighting of dominant species) using the Bray–Curtis dissimilarity algorithm. Nonmetric multidimensional scaling (MDS ordination) was used to investigate the effect of different soil treatments on the structure of the bacterial and fungal communities; on MDS plots and cluster analysis, most similar communities are grouped closer together (Clarke & Warwick, 2001). Two-way (stubble by nitrogen) crossed analysis of similarities (ANOSIM) was performed on the similarity matrix data set (Bray–Curtis). Redundancy analysis (RDA) was used to relate environmental log-transformed variables to microbial community structure, with the Monte Carlo permutation test used to calculate the significance and proportion of the total variance in community structure explained by environmental variables (CANOCO 4.53; Microcomputer Power).

Data transformation, MDS, ANOSIM and diversity measures were carried out in PRIMER5 software (Primer-E Ltd). Selected ecological indices were calculated as described in Kennedy & Smith (1995): Shannon's diversity index (H'), Margalef's richness index (d) and Pielou's evenness index (J').

$$H' = -\sum P_i \log P_i, \quad d = \frac{(S-1)}{\log(\mathrm{N})}, \quad J' = \frac{H'}{\log(S)}$$

where S = total number of species, N = number of individuals and P_i = proportion of the total count arising from the ith species. ANOVA was used to analyse the effect of management treatments, soil physicochemical properties and functional gene abundance (GenStat8; Lawes Agricultural Trust).

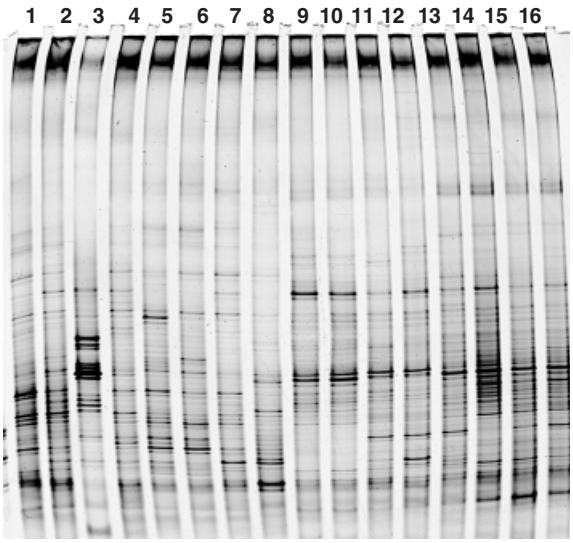

Fig. 1. Fungal DGGE gel showing profiles of ITS1 region of the rRNA genes amplified from soil using PCR. Each band is inferred to represent a distinct operational taxonomic unit, and band intensity is inferred as a measure of abundance. Lanes 1–4 = stubble burnt, no N application; lanes 5–8 = stubble burnt, +N fertilization; lanes 9–12 = stubble retained, no N application; lanes 13–16 = stubble retained, +N fertilization.

Results

Soil physicochemical properties and microbial biomass

Retention of maize stubble significantly increased soil total C, organic C and total N, but decreased NO_3^- N (Table 2). The only significant effect of application of N to soil (329 kg N ha^{-1} year^{-1}) was an increase in N as NO_3^-. The major treatment factor affecting MBC was stubble retention, which led to a significant increase ($P < 0.001$) (Table 2). Nitrogen application reduced MBC where stubble was retained ($P = 0.011$). The amount of DNA extracted from soil increased with stubble retention ($P < 0.001$), but not with N addition (Table 2). Overall, there was a strong correlation between extractable DNA and MBC ($R^2 = 0.79$).

Microbial community structure and diversity

Stubble retention and nitrogen application significantly ($P < 0.05$) affected the structure of the bacterial and fungal communities (Fig. 1; ANOSIM; Table 3). *R*-values show that for both microbial communities, the strongest discriminator of community structure was stubble retention (Table 3). These results were supported by MDS analyses, which again

Table 2. Soil physicochemical properties and microbial population size under different stubble and N management systems

Treatment	Total C (%)	Organic C (%)	Total N (%)	NH_4^+* (mg kg^{-1})	NO_3^-* (mg kg^{-1})	Total P (mg kg^{-1})	Ext-P† (mg kg^{-1})	c.e.c.‡ (cmol(+) kg^{-1})	MBC§ (mg C kg^{-1})	DNA¶ (ng g^{-1})
Stubble burnt, −N	1.199	1.135	0.116	6.1	17.6	598	97.9	26.64	91.0	209
Stubble burnt, +N	1.271	1.205	0.128	31.7	43.1	621	133.0	27.83	93.1	207
Stubble retained, −N	1.550	1.494	0.133	7.9	4.3	610	113.1	26.86	224.7	409
Stubble retained, +N	1.548	1.548	1.135	6.3	18.5	569	106.8	28.15	161.1	502
P values										
Stubble	0.002	0.002	0.040	0.198	< 0.001	0.633	0.640	0.656	< 0.001	< 0.001
Nitrogen	0.648	0.456	0.193	0.189	< 0.001	0.825	0.234	0.064	0.016	0.329
Stubble × Nitrogen	0.629	0.924	0.357	0.142	0.075	0.435	0.101	0.931	0.011	0.310

*KCl-extractable.
†HCO_3^--extractable P (Colwell, 1963).
‡Cation exchange capacity.
§Microbial biomass carbon.
¶DNA extracted from three 1-g samples of soil.

Table 3. Two-way crossed ANOSIM of effect of stubble and nitrogen management on bacterial and fungal community structure

Microbial community	Treatment	*R*-statistic*	*P*-value
Bacteria	Stubble	0.760	0.001
	Nitrogen	0.292	0.030
Fungi	Stubble	0.639	0.001
	Nitrogen	0.573	0.003

*For the null hypothesis, $R = 0$ (no discrimination of population between treatments). Potential range of $R = -1$ to 1.

showed that both bacterial and fungal communities differed in composition under stubble burnt and stubble retention regimes (Fig. 2a and b). The structure of the fungal community was also affected by nitrogen addition ($R = 0.573$, $P = 0.003$), but on the MDS plot it is evident that this effect was secondary to stubble treatment (Fig. 2b). Cluster analysis (complete linkage) showed similar effects (data not shown).

Redundancy discriminate analysis (RDA ordination) with Monte Carlo permutation was used to relate environmental variables with microbial community structure. For the bacterial community, RDA described 43.5% of the variation within the first two axes (Fig. 3a), and for the fungal community 39.1% (Fig. 4a). The low explanation of variation over two dimensions was due to the large species numbers (43 OTUs for bacteria; 59 fungal). Nevertheless, the bacterial community structure clearly separated along the *x*-axis based on stubble treatment (Fig. 3a). Variation in soil carbon (total or organic) explained 12–13% of the changes in bacterial community structure ($P < 0.05$; Table 4, Fig. 3b). Total N, NO_3^-, K^+ and Na^+ were also significantly related to changes in bacterial community composition ($P < 0.05$; Table 4, Fig. 3b). Similarly, variation in fungal community composition was most strongly linked to changes in soil total and organic C, NO_3^- (but not total N) and K^+ (Table 4, Fig. 4b).

Both stubble retention ($P = 0.001$) and nitrogen addition ($P = 0.012$) increased the richness of fungal, but not bacterial, DGGE banding patterns (Fig. 1, Table 5). Neither the evenness nor the diversity of fungal and bacterial community profiles was significantly affected by stubble or N addition (Table 5).

Functional gene detection

The cycle numbers at which exponential PCR amplification (C_T) of key genes for nitrogen fixation (*nif*H) and denitrification (*nap*A) were reached were significantly lower in soil in which stubble had been retained (Table 5). As C_T is inversely proportional to DNA template, relative abundance of DNA template (copy number) for both the *nif*H and *nap*A genes was significantly higher under stubble retention. Conversely, C_T for *amo*A detection was not affected by stubble retention, but by nitrogen application (Table 5). With applied N, the C_T for *amo*A detection was significantly lower than for soils with no N application ($P < 0.003$). Therefore, N application significantly increased the copy number of *amo*A genes in the maize soils. The copy number of *nif*H, the gene for nitrogen fixation, increased in abundance by a factor of 2.2 under stubble retention. This takes into account a 4.2-fold increase in *nif*H copy number per × 1.9 increase in total microbial population following stubble retention.

Discussion

Changes in farm management practices have a direct impact on soil microbial community structure, thereby affecting processes such as the biogeochemical cycling of major elements (e.g. C, N, P), decomposition processes, disease

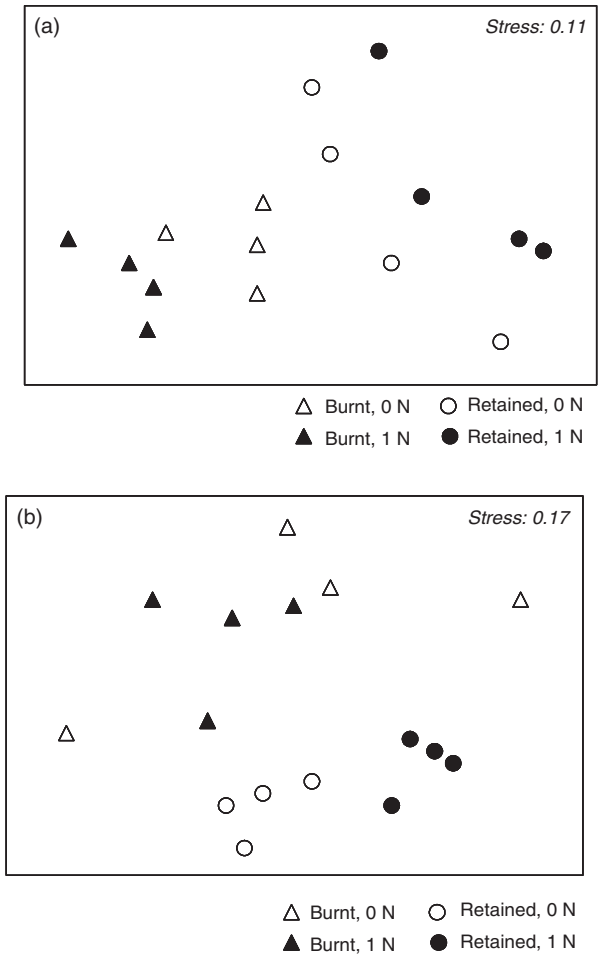

Fig. 2. Nonmetric multi-dimensional scaling analysis, of the effect of stubble and N management on soil (a) bacterial and (b) fungal communities. Stubble either retained or burnt; nitrogen either added or not.

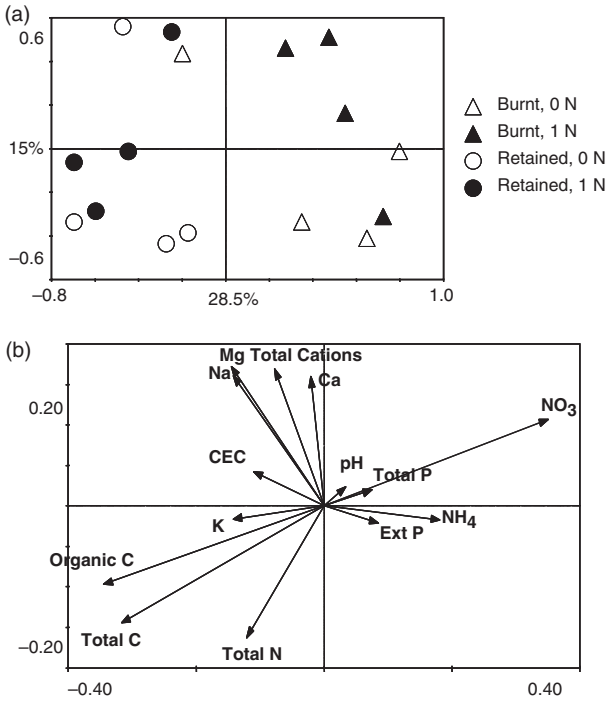

Fig. 3. Output of redundancy discrimination analysis relating environmental variables bacterial community structures in soils with differing stubble and N management treatments. (a) Ordination plot of bacterial community structures based on 16S-rRNA gene PCR-DGGE profiles. (b) Environmental factors associated with changes in the bacterial community structure; direction of arrow indicates which biological factors are associated with the environmental parameter and the length of the arrow indicates the magnitude of the association.

suppression and other ecosystem functions (Swift & Anderson, 1994). Such process changes have been observed following retention of maize stubble and application of N-based fertilizer in a high-value irrigated production system in Australia (Edis *et al.*, 2006; Harvey *et al.*, 2006; Kirkby *et al.*, 2006; Meyer *et al.*, 2006). Using soil from the same site, we have shown a significant change in the size of the total microbial community and in the structure of fungal and bacterial communities between management treatments. Using functional gene-based quantitative PCR, we also showed that agricultural management practices at the site directly affected the abundance of key groups of N-cycling bacteria. As such, this work supports previous evidence for an enduring change in the microbiology of the soil, which is linked to observed and measured changes in microbially driven processes and which has importance for both production and environmental endpoints.

Successive years of maize stubble retention and N addition to soil resulted in significant alteration in soil physico-chemical and biological properties, even when determined at the end of the winter fallow period. With stubble retention, levels of total and organic C significantly increased in the soil, as did the size of the soil microbial community (as determined by MBC and extractable DNA). Although there was a strong correlation between the amount of DNA extracted from soil and MBC ($R^2 = 0.79$), the actual usefulness of using extracted DNA concentration as a method of quantifying microbial biomass between soils is questionable owing to variation in efficiencies of DNA extraction between soil types and between techniques used (e.g. Miller *et al.*, 1999). Furthermore, it is likely that some of the DNA extracted from the soil would have originated from nonmicrobial material, such as plant residues and soil micro- and macrofauna.

Stubble retention also increased soil total N, but mineral N (NH_4^+) levels remained unchanged. Nitrogen as NO_3^- was significantly higher where stubble was burnt and where N was applied. Some of the N in the stubble-retained soils was held in increased microbial biomass (MBN). In stubble retention, MBN increased from 20.4 to 34 mg N kg soil^{-1} ($P = 0.064$), suggesting that some of the N

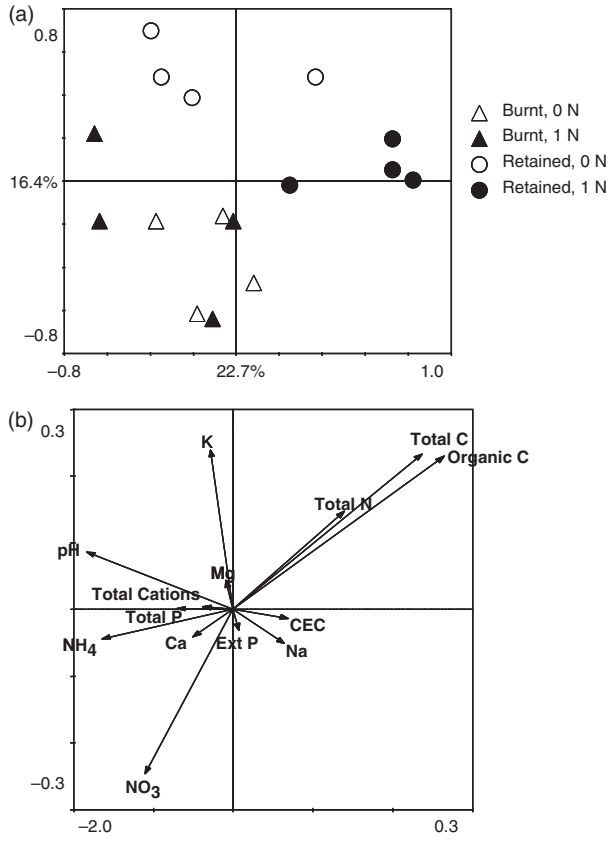

Fig. 4. Output of redundancy discrimination analysis relating environmental variables to fungal community structures in soils with differing stubble and N management treatments. (a) Ordination plot of fungal community structures in bases on ITS-rRNA gene PCR-DGGE profiles. (b) Environmental factors associated with changes in the fungal community structure, direction of arrow indicates which biological factors are associated with the environmental parameter and the length of the arrow indicates the magnitude of the association.

in the stubble-retained soil system was incorporated into the increased microbial biomass. The microbial biomass was negatively affected by N addition in the stubble retention treatments.

Stubble retention and N addition were shown to influence community structure significantly, as determined using PCR-DGGE profiling. Analysis of both bacterial and fungal communities demonstrated that stubble retention was the strongest driver affecting species composition, particularly for the fungal community. Using PCR-DGGE, Clegg *et al.* (2003) found that N fertilizer exerted a significant effect on the total bacterial and actinomycete community structures. Similarly, we found that nitrogen application affected total bacterial and fungal community structure, but the effect of N was not as strong as that of stubble management. RDA relating bacterial and fungal community structure to changes in soil physicochemical properties explained $< 50\%$ of the variation. As such, other environmental drivers not measured in this study were important in driving microbial community structure. Nevertheless, changes in bacterial and fungal communities were correlated with changes in several soil physicochemical properties. Not surprisingly, changes in soil C and N (total or NO_3^-) accounted for the largest amount of variation in terms of community composition. Changes in K and Na explained low but significant portions of the variation in the bacterial and fungal communities. Concentrations of cations were higher in stubble-burned treatments owing to the deposition of oxidized ions back into the soil (i.e. K, Na, Ca and Mg oxides) (Xu *et al.*, 2002).

Table 4. Percent variation in microbial community composition (PCR-DGGE) explained by environmental factors

	Bacterial community	Fungal community
Organic C	13.0*	12.6*
Total C	12.0*	11.7*
NO_3^-	13.0*	9.7*
K	8.7*	9.7*
Total N	6.9*	
Na	6.9*	

Results from Monte Carlo permutation test (unrestricted permutations). *$P < 0.05$.

Interestingly, the effect of agricultural management practice was greater for the fungal community than the bacterial. Previously, similar studies have focused on the effects of farm management processes on the soil bacterial community. The major drivers affecting structure of total bacterial communities are soil type and plant species (Girvan *et al.*, 2003; Garbeva *et al.*, 2004) although many other factors may have significant influence as shown in this study. The fungal community also appears to be highly responsive to ecosystem change, particularly in relation to high rates of organic inputs such as stubble. Soil fungi play important roles in cycling of C, denitrification and production of N_2O (e.g. Shoun *et al.*, 1992), they elicit and suppress plant disease, and stabilize soil aggregates, etc. Further studies on management effects on microbial communities should therefore include both bacteria and fungi.

Although stubble and N treatment significantly affected microbial community size and structure, very little effect was observed on diversity estimates. This lack of effect was surprising, as repeated addition of a large amount of maize stubble ($10\,t\,ha^{-1}$) might be expected to increase the community size and dominance of specific fungal decomposers, thus decreasing richness and evenness. However, the richness of the fungal community significantly increased following both application of stubble or N to the soil. It is important to recognize the limitations of ribosomal-based PCR-DGGE when studying microbial diversity. In particular, assessment of band numbers can be a poor surrogate for diversity owing to comigration of bands, multiple bands

Table 5. Effect of stubble and N management practices on ecological indices of soil bacterial and fungal communities and functional gene abundance

	Richness *d*		Evenness *J′*		Diversity *H′*		C_T for functional gene PCR*		
Treatment	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	*nif*H	*amo*A	*nap*A
Stubble burnt, −N	1.47	1.44	0.86	0.81	2.66	2.47	16.46	29.70	22.24
Stubble burnt, +N	1.69	1.29	0.88	0.67	2.84	1.98	16.90	27.76	21.86
Stubble retained, −N	1.86	1.42	0.83	0.76	2.75	2.33	14.94	30.05	19.85
Stubble retained, +N	2.35	1.31	0.86	0.82	3.07	2.47	14.31	28.11	10.02
P values									
$P_{(stubble)}$	0.001	0.987	0.357	0.743	0.217	0.732	< 0.001	0.491	< 0.002
$P_{(nitrogen)}$	0.012	0.084	0.420	0.834	0.071	0.742	0.780	0.003	0.085
$P_{(interaction)}$	0.273	0.800	0.857	0.542	0.613	0.550	0.080	0.992	0.174

*C_T (dRn) of amplification following real-time PCR; threshold at which exponential amplification occurred.
Lower C_T, greater target template copies.

from single species owing to heterogeneity in rRNA gene sequences and amplification bias of PCR. For example, total band positions for the bacterial communities only delimited 43 different OTUs. Given the huge overall diversity of bacterial species in most soils, it is clear that the approach only assesses numerically dominant species.

Significant shifts in functional diversity (substrate utilization) following addition of maize stubble or litter to soil have been demonstrated. Using Biolog® GN plates, Sharma *et al.* (1998) showed that functional diversity of soil bacterial communities increased after addition of maize litter in several different soils. In their incubation experiment, the shift in function was measurable over 32 weeks after maize litter addition. In the present study, we have shown that changes in microbial community size and structure, as well as the abundance of key functional genes, are affected by management of stubble and N into soil. Measurements were made from field soil following the winter fallow period, demonstrating the enduring effect of management treatments and the potential for subsequent impact on the following crop.

At the Whitton field site, the geochemical cycling of N has been studied in detail (Edis *et al.*, 2006; Meyer *et al.*, 2006). In particular, research has focused on the production of N_2O as a result of either denitrification or nitrification, the latter being a relatively minor process in terms of total N_2O emissions. It is generally assumed that a microbial community capable of undertaking these processes is both present and potentially active under the given agroecological conditions. PCR amplification of genes involved in nitrate reduction (*nap*A) and nitrification (*amo*A) was used to demonstrate the microbial capacity for these processes to occur in the soil and showed that the management of stubble and N had a direct effect on the population size of N-cycling bacteria. Under stubble retention, the population size of denitrifying bacteria was much higher than under the stubble-burnt treatment. Although this is inconsistent with the increased N_2O emissions from the stubble-burnt treatment, the results are consistent with denitrification potential of the soil if the physicochemical properties at the time of sampling are considered. At the end of the fallow period, soil NO_3^- concentration was significantly greater following both stubble retention and N fertilization than in stubble retention only, but there was no significant difference in soil NH_4^+. The genetic capacity to reduce NO_3^- is common to a wide range of soil bacteria, including nonspecialized denitrifying bacteria. As such, an increase in the population size of nitrate-reducing bacteria may be the result of greater overall soil microbial population size in the stubble-retained treatment. To assess the microbial potential for N_2O emissions in a soil system accurately, other genes involved in the denitrification pathway should be also assessed.

In contrast to the abundance of the denitrification gene *nap*A, the size of the microbial community involved in nitrification was affected only by nitrogen addition. This result is not surprising as ammonia-oxidizing bacteria (AOB) are chemolithotrophic. Although the size of the AOB community is dependent on NH_4^+, the ecophysiological preferences among AOB means that their activity can be affected by ecosystem change. Thus, agricultural practices, such as stubble retention, can potentially affect AOB communities and impact on N-cycling until a new community structure, adapted for the current environmental conditions, is stabilized.

The abundance of free-living N_2-fixing bacteria approximately doubled following stubble retention but was not affected by N application. Large inputs of C (e.g. maize stubble) to soil significantly increase the abundance of free-living N_2-fixing bacteria (Hegazi *et al.*, 1986). However, it is also generally accepted that application of high rates of N to soils reduces dependence of the ecosystem on free-living N_2-fixing organisms. This discrepancy may be explained by considering the timing of stubble addition to the soil and the soil sampling for this study. Maize stubble was incorporated into the soil at the end of the growing season by which time some of the N applied over the growing season had been

removed by either biotic or abiotic processes. Thus, the decomposition of stubble during the winter fallow period may still be enhanced through the activity of N_2-fixing bacteria. Although the contribution of free-living N_2-fixing bacteria to N-uptake of maize in high-input agricultural sites is likely to be very low, it is clear that these bacteria have ecological importance other than provision of N for plant use. In particular, the activity of these bacteria over the winter fallow period may aid in the decomposition of organic waste with high C : N ratio.

The results of the real-time PCR allowed quantification of functional gene presence under the different soil treatments; absolute quantification of copy number of these genes was not readily achievable in this study. A major problem is that different species of bacteria can hold varying copy number of each functional gene. Nevertheless, the data clearly show that modification of both stubble and N management practices has a significant impact on the overall quantity of bacterial genes involved in key steps of the N cycle.

Establishing links between bacterial or fungal diversity and ecosystem processes in soil is difficult. Studies reporting strong associations between these factors are rare and typically focus on highly stressed or disturbed ecosystems (e.g. Girvan *et al.*, 2005). Underpinning these studies are ecological hypotheses suggesting that due to distribution of functionality traits across broad phylogenetic groups of soil microorganisms, associations between soil microbial diversity and ecosystem processes (function) are tenuous. However, spatial and temporal environmental heterogeneity in soil may require that a multitude of species with physiological variability and habitat preferences occur together for long-term ecosystem stability. This is important for the maintenance of strong ecological resistance to single or multiple stresses (whether natural or anthropogenic).

This work has shown that long-term management of maize stubble and N had significant post-seasonal impacts on the size and structure of bacterial and fungal communities in soils. Such sustained shifts in soil microbial communities can impact on ecosystem function, as shown by overall changes in N-based functional genes, and also on the productivity of a subsequent crop. Future work will focus on the relationship between the structure of ecologically significant functional communities and ecosystem processes at this trial site.

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