Pasture management clearly affects soil microbial community structure and N-cycling bacteria

Steven A. Wakelin\textsuperscript{a,*}, Adrienne L. Gregg\textsuperscript{a}, Richard J. Simpson\textsuperscript{b}, Guandgi D. Li\textsuperscript{c}, Ian T. Riley\textsuperscript{d}, Alan C. McKay\textsuperscript{d}

\textsuperscript{a}CSIRO Land and Water, PMB 2, Glen Osmond, South Australia 5064, Australia
\textsuperscript{b}CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia
\textsuperscript{c}E H Graham Centre for Agricultural Innovation (Alliance between NSW Department of Primary Industries and Charles Sturt University), Wagga Wagga Agricultural Institute, PMB, Wagga Wagga, NSW 2650, Australia
\textsuperscript{d}SARDI Plant and Soil Health, GPO 397, Adelaide, South Australia 5001, Australia

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\textbf{Summary}

There may be significant benefits to understanding and managing microbial processes in pasture soils. Direct benefits include increased production, but microbial processes also affect the sustainability and environmental impact of production systems. The first step towards achieving such gains is identifying the components of the soil microbial community most responsive to pasture management. We used molecular tools (DGGE and TRFLP) to assess the impacts of pasture type, grazing, liming, P fertilisation and sampling date on the structure of the soil bacterial and fungal communities. Furthermore, the response of bacteria involved in defined ecosystem processes was determined by quantifying genes (qPCR) involved in nitrogen fixation (\textit{nif}\textsubscript{H}), nitrification (\textit{amo}\textsubscript{A}) and denitrification (\textit{nar}\textsubscript{G}). The strongest factor affecting the structure of the soil microbial community was liming of acidic soil at the Book Book (New South Wales) field trial site ($R^2 = 0.95; P < 0.001$). Effects of pasture type (annual vs. perennial) were minor. Addition of lime (management of soil pH) increased the richness of fungal phylotypes (Margalefs index; $P = 0.017$) but not bacterial phylotypes. Liming also increased the biological capacity for nitrogen fixation and nitrification ($P < 0.05$). Across all treatments, there was a very good correlation between previous measures of N\textsubscript{2} fixed at the site and \textit{nif}\textsubscript{H} gene abundance data ($R^2 = 0.81$). The biological capacity for denitrification did not respond to pasture management. At a field site near Hall (Australian Capital Territory), an increase in the intensity of pasture production by phosphorus (P) fertiliser addition or increased stocking rate also affected the structure of the soil fungal and bacterial communities ($R = 0.8; P < 0.001$) and increased fungal...
phylotype richness ($P<0.05$). Significant shifts in the soil biota also occurred during the growing season ($P = 0.001$). These observations may have been strengthened by the onset of drought conditions from July 2006. Richness of fungal, but not bacterial phylotypes, increased with P fertilisation, stocking rate, and between sampling times ($P<0.05$). The increase in fungal phylotypes richness was in direct contrast to a reduction in botanical diversity. Temporal variation in fungal richness was positively correlated with monthly rainfall ($R^2 = 0.774$). The biological capacity for nitrification and denitrification increased with intensification of the system ($P<0.05$), but the biological capacity for $N_2$ fixation increased with stocking rate only ($P<0.05$). Overall, results showed that soil biota under pastures, and particularly soil fungi, are highly responsive to agricultural management treatments.

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Introduction

Soil microorganisms and their associated processes underpin many essential ecosystem functions. These include biogeochemical cycling of most elements essential to plant health and ecosystem fertility, causing and controlling plant disease, mycorrhizal and rhizobia symbiosis with plants, decomposition of animal, plant, and chemical waste, and soil physical structural formation and stability (reviewed in Roper and Gupta, 1995). Many of these microbially mediated processes are directly impacted by farm management activities (Roper and Gupta, 1995; Bünemann et al., 2006). As such, there is potential to manage soil biology and processes for both production and environmental gains (Gupta and Ryder, 2003).

Pasture-based production systems are inherently complex and subject to a wide range of management practices. These include pasture species composition, plant density and pasture renovation, grazing intensity, crop transitions, fertiliser and pesticide inputs, irrigation and soil improvement (e.g. liming or soil deep ripping). Not surprisingly, soil microbiology and biological processes within these pasture-based agri-ecosystems are complex and poorly understood (Gupta and Ryder, 2003), and knowledge gains have been hampered by technological limitations. Most research to date has therefore been based at organism level or focused on single processes. Despite this, the benefits gained from understanding soil biology have already been of immense benefit to the pasture industry, significant examples being research into rhizobia–pasture legume interactions (e.g. Evans et al., 2005; Howieson et al., 2000) and plant diseases (e.g. Ballard et al., 2006; Barbetti et al., 2006).

Future directions for soil microbial research in pasture systems include achieving productivity gains through enhanced biological cycling of soil nutrients such as C, N and P (Gupta and Ryder, 2003). In addition, there is interest in managing biological functions to increase the sustainability of ecosystem processes, for example by increasing ecosystem stability (resilience and resistance, sensu Orwin and Wardle, 2004). It is also desirable to reduce the environmental impact of microbial processes, in particular those associated with production of greenhouse gasses such as $N_2O$ and $CH_4$ (Dalal et al., 2003), and increase the biodiversity (biological capacity) of soil biota responsible for key ecosystem functions (Coleman and Whitman, 2005). To capture such gains, approaches based at a community level and those incorporating functional microbial ecology must be taken. These approaches can be aided through the application of modern techniques, in particular molecular microbial ecology approaches (van Elsas et al., 1997).

The aims of this study were to determine how responsive various components of the soil microbial community were to a range of different pasture-based land-use practices in southern Australia. In addition to characterising community shifts across broad phylogenetic groups (bacteria and fungi using PCR-TRFLP and PCR-DGGE, respectively), molecular approaches were used to quantify the abundance of functional groups involved in N cycling (real-time PCR). The work was conducted towards identifying the most responsive components for future work and not, at this stage, an attempt to relate specific soil or ecosystem processes to microbial communities per se. In order to encompass a broad range of pasture land-use types within an actual operating land-use context, two long-term, replicated field trials were examined. Together, treatments across these trials varied in management of P fertiliser addition, pasture composition, grazing levels, liming application and sampling date.
Materials and methods

Book Book site

The Book Book site is in south-east New South Wales (NSW; 35°12’S, 147°18’E) and receives annual rainfall of 650 mm. The soil is a subnatric yellow sodosol with some red phases over the site (Isbhill, 1996). The average pH in 0.01 M CaCl2 (pHc) of the 0–10 cm soil depth was 4.0 and subsurface pHc was below 4.5 to at least 20 cm, which was typical of the more acidified soil in the region.

Two types of pastures (perennial vs. annual pastures) with or without lime application were established in 1992, and re-sown with original mixes in 2004 as part of the Managing Acid Soils Through Efficient Rotations (MASTER) experiment. The original experimental design consisted of 2 blocks of 40 plots with 8 treatments (Li et al., 2001). The current study was conducted on 4 continuous pasture treatments only, i.e. perennial pastures with and without lime application, and annual pastures with and without lime application. Four plots from each treatment were selected as test plots. There were 16 plots in total with plot size of 30 × 45 m.

The perennial pasture was sown to phalaris (Phalaris aquatica L.) cvw. Australian and Holdfast, cocksfoot (Dactylis glomerata L.) cv. Currie, lucerne (Medicago sativa L.) cv. Aurora and subterranean clover (Trifolium subterraneum L.) cvw. Junee, Goulburn and Trikkala. The annual pasture was sown to annual ryegrass (Lolium rigidum Gaudin) cv. Wimmera and subterranean pasture was sown to annual ryegrass (cvv. Junee, Goulburn and Trikkala. The major weeds on the site were clover cvv. Junee, Goulburn and Trikkala. The weed species in the site were Vulpia spp., barley grass (Hordeum leporinum Link), sorrel (Rumex acetosella) and capeweed (Arctotheca calendula). Pastures were rotationally grazed over the past 14 years from 1992 to 2006.

The initial lime (3.7 t/ha) was incorporated into the 0–10 cm soil depth and the maintenance lime (1.8 t/ha) was top-dressed at 6-year intervals to maintain average pHc at 5.5 in the 0–10 cm soil depth. Phosphorus (P) and potassium (K) were applied at 15 and 25 kg K/ha every year, respectively. Molybdenum (Mo) was applied at 50 g Mo/ha every 5 years. Further details on site description and pasture management were described in Li et al. (2001, 2006a, b) and White et al. (2000).

Hall site

The site is located on the Wallaroo-3 paddock trial of CSIRO’s Ginninderra Experiment Station near Hall, Australian Capital Territory (ACT; 35°06’S, 149°03’E). A P fertilisation × grazing (stocking rate) experiment was established in 1994 on Yellow Chromosol soil (Isbhill, 1996) with pH(CaCl2) of 4.6 in the topsoil (0–10 cm depth). Pasture treatments were grazed continuously by either 9 sheep/ha (0.66 ha plots) or 18 sheep/ha (0.33 ha plots). At the establishment of the trial, the site was deficient in P for plant growth with surface soil HCO3-extractable P (Olsen, 1954) of 4 mg/kg soil. Basal nutrients (Ca, S, K, Mo, B and Zn) were applied to ensure adequate supply for plant growth. The P treatments were either unfertilised, or fertilised with differing amounts of triple superphosphate (21% P) or superphosphate (9% P) each autumn to achieve a target Olsen P of 10–12 mg/kg soil during winter/early spring (Hill et al., 2004). Over the period 1994 to April 2006 (when soil sampling for the present experiment began) a total of 205.5 or 235.8 kg P/ha had been applied to the fertilised low stocking rate or higher stocking rate treatments, respectively. A further 8.2 and 3.3 kg P/ha were applied to these treatments, respectively, in May 2006 during the sampling period. The fertiliser × grazing treatments generated significant differences in the botanical composition of the pasture such that by 1999–2001, for the main pasture species, the most abundant annual grass on unfertilised plots was Vulpia spp., whilst that on the fertilised plots was Bromus spp. Fertiliser and the low stocking rate promoted cover by Phalaris aquatica (L.), but depressed Trifolium subterraneum. Fertiliser and the high stocking rate promoted T. subterraneum (L.) cover. Further details of the Hall site experiment and its botanical composition are reported by Hill et al. (2004). Fertilised and non-fertilised plots at stocking rate 9, and fertilised plots at stocking rates of 9 and 18 were sampled monthly from April 2006 until September 2006 (6 samples). Three replicate field plots from each treatment were sampled.

Soil sampling, DNA extraction and quantification

Soil was sampled using a 10 × 100 mm corer. From each field plot, 30 cores were sampled in a “W” transect and a composite sample of around 350–400 g, representative of the plot was generated. The composite samples were stored on ice and transported back to the laboratory. The soils were coarse-sieved to remove plant trash, stones, etc., and then freeze-dried and then processed for DNA extraction via a commercially available service.
Bacterial community profiling

The structure of soil bacterial communities was profiled using TRFLP (Rasche et al., 2006). 16S-rRNA genes were amplified from soil-extracted DNA using the primers 8F and 1520R. PCR conditions were as described in Rasche et al. (2006), but the chemistry was based on Qiagen HotStar Taq polymerase and buffer system. The forward primer was labelled with FAM and the reverse primer HEX (oligonucleotides were synthesised by Geneworks Pty Ltd., Adelaide, Australia). PCR product was purified from the reaction mix using a commercial clean up kit in 96 well plate format (MoBio Inc) and 10 µl digested using AluI and CfoI. Products were checked for full digestion using agarose gel separation. The size of fluorogenically labelled terminal restriction fragments (both ends labelled using Fluorogène) were measured via capillary separation (Australian Genome Research Facility, Adelaide) against known oligo-size fragments (LIZ500[-250] marker; ABI). Size and intensity data were assessed using GeneMapper software (ABI); only peaks with intensities of ≥ 100 fluorescent units were included for analysis.

Fungal community profiling

Fungal community structures were measured via denaturing gradient gel electrophoresis (DGGE) of the rRNA-ITS region. The methodology followed was that described in Wakelin et al. (2007) using primers ITS1F-GC and ITS2*; however, DGGE separation was carried out in an Ingeny PhorU system (Ingeny International, The Netherlands). The polyacrylamide gels (7% w/v of acrylamide:bis-acrylamide at 37.5:1) contained a linear formamide/urea gradient ranging from 20% to 50% and were overlayed with a non-denaturing stacking gel. Each well received 10 µl of PCR product and 4 µl of 2 × loading buffer. Electrophoresis was conducted at 110 V for 17 h, after which the gels were stained with SYBR Gold (Molecular Probes) for 40 min, rinsed briefly in water, and visualised on a Dark-Reader (Clare Chemical Inc). An Olympus E500 SLR digital camera was used to photograph stained gels. Position and intensity of bands on the DGGE gels were measured using Gel-Quant software (Multiplexed Biotechnologies Inc.).

Functional gene quantification

Functional genes encoding key enzymes involved in nitrogen fixation (nifH), nitrification (amoA) and nitrate reduction (narG) were quantified using real-time PCR. PCR amplification of nifH followed the method of Rösch et al. (2002). narG detection was based on Philippot et al. (2002) and quantification of the amoA gene based on the primers of Stephen et al. (1999) and Rotthauwe et al. (1997). Amplification was performed in 25 µl reaction mixes using the QuantiTect SYBR Green PCR kit (Qiagen) on an MX3000P q-PCR system. Final reaction concentrations each used 0.8 µM of forward and reverse primers, 2 µl of undiluted DNA sample, 12.5 µl of 2 × PCR master mix (including Taq DNA polymerase, SYBR, etc.) and water total volume of 25 µl. PCR cycling conditions were as follows: 40–50 cycles of 95°C for 45 s, primer annealing for 1 min at 60°C for nifH, or 50°C for amoA, or 55°C for narG, and template extension at 72°C for 45 s. The reactions were run for 40+ cycles until no further amplification of samples was observed. The threshold cycle (Ct) in which all reactions were in exponential phase amplification was software calculated and did not require manual adjustment. Functional genes can vary in oligonucleotide composition between bacterial species which may affect melting (dissociation) curve analysis on q-PCR systems. Therefore, to ensure amplification of specific PCR product, reaction mixes were separated by agarose electrophoresis and stained using ethidium bromide.

Standard curves relating calculated DNA amounts and Ct values were used to quantify copy numbers of each gene. PCR products from each gene were cloned into the TOPO-TA cloning vector (Invitrogen). Plasmid DNA containing a single gene copy was extracted using the MoBio plasmid prep kit and quantified using Picogreen as before. Ten-fold serial dilutions of the DNA from the plasmid were prepared to obtain a standard curve for each gene spanning eight orders of magnitude. Each standard curve was run in duplicate. Copy numbers for each sample were calculated (using known sequences of the vector and PCR insert) and the plasmid concentrations, expressed in units of copy numbers per µg DNA, extracted to define the relative abundance of the targeted bacterial functional genes present within the DNA associated with the total microbial community.
Data analysis

Similarities between community structures were determined using the Bray–Curtis algorithm on 4th root transformed abundance data. The richness of fungal and bacterial phylotypes (SSU rRNA genotypes) were measured using Margalefs’ index \( d; \text{ Eq. (1)} \), as described in Kennedy and Smith (1995):

\[
d = \frac{(S - 1)}{\log(N)}
\]

(1)

where \( S \) is the number of phylotypes (resolvable DGGE bands or TRFLP peaks) and \( N \) the band intensity or peak height.

Non-metric multidimensional scaling (MDS; Clarke, 1993) was used to assess the similarity of microbial communities across field treatments. The effect of treatment on microbial community structure was tested using one- or two-way ANOSIM (Clarke, 1993). At the Hall site, the effects of P fertiliser addition could only be determined at a stocking rate of 9, and the effects of stocking rate could only be assessed at P application rate of 1.

Multivariate statistical analysis was carried out using the Primer6 software package (Primer-E, Warwick, UK) following methods described in Clarke and Warwick (2001).

The effects of liming and pasture type on the abundance (copy numbers/ng DNA) of N-cycling functional genes were assessed by two-way ANOVA. At the Hall site, the effects of P fertilisation and stocking rate on the abundance of \( nifH \), \( amoA \) and \( narG \) were assessed using Mann–Whitney (non-parametric) two-tailed \( t \)-tests with 95% confidence intervals. The effects of sampling date on functional gene abundance were determined using Friedmans one-way ANOVA (repeated measures, non-parametric). Post-hoc pair-wise comparisons were made between all samples using Dunn’s test at \( \alpha = 0.05 \). Analysis of functional gene data was carried out using Prism Version 5.00 for Windows (GraphPad Software, San Diego, USA).

Results

Book Book site

Strong rainfall events had occurred at the Book Book site in the month leading up to sampling in July, 2006 (Figure 1A). This rainfall had followed an extended dry and hot summer drought period (Figure 1A).

Soil bacterial and fungal communities varied between treatments at the Book Book site (Figure 2).

Application of lime to pasture soils significantly increased the abundance of \( nifH \) by 29%
(P = 0.041), and amoA genes by 26% (P < 0.001), but had no effect on narG copy numbers (Figure 3). There were no differences in the abundance of N-cycling genes between perennial or annual pastures (P > 0.05), nor was there an interaction between pasture and liming treatments (P > 0.05).

Hall site

Sampling at the Hall site occurred over the months of April to September, 2006. Monthly rainfall varied considerably over this period (Figure 1B), with drought being a dominant influence. Rainfall was below average until June, when pasture growth recommenced. By September, plant-available moisture was again becoming limiting and a spring drought was experienced. The onset of drought into the sampling period affected pasture growth and most probably the below ground microbial processes.

The structure of the soil fungal community was highly responsive to pasture management practices (Table 1). Strong shifts in the community occurred with addition of P fertiliser (R = 0.833; Table 1) and increased stocking rate (R = 0.79; Table 1). These changes in community structure are shown through
Table 1. Summary of effects of phosphorus fertiliser addition, stocking rate and sampling time on the fungal and bacterial community structures in soil from the Hall field site.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fungal community</th>
<th>Bacterial community</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Global R</td>
<td>P value</td>
</tr>
<tr>
<td>Phosphorus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.833</td>
<td>0.001</td>
</tr>
<tr>
<td>Time</td>
<td>0.528</td>
<td>0.001</td>
</tr>
<tr>
<td>Stocking rate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.790</td>
<td>0.001</td>
</tr>
<tr>
<td>Time</td>
<td>0.762</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Effect of phosphorus fertiliser addition assessed at stocking rate of 9 only.

<sup>b</sup>Effect of stocking rate assessed at P addition rate of 1 only.

Figure 4. MDS plot showing resemblance of soil fungal community structure (PCR-DGGE) as affected by P fertiliser addition and stocking rates at the Hall field site over the April to September period, 2006.

the grouping of the fungal community structures with management treatments in Figures 4A and B. Significant changes in the fungal community also occurred between sampling dates (Table 2; Figure 4). Pair-wise comparisons showed monthly shifts in community structure when stocking rate was the main factor (Table 2). Between the P fertiliser treatments, the magnitude of the temporal shifts was lower than for stocking rates (Global R values; Table 2). Notably, the fungal community in soil sampled during April was significantly different to all other months (P<0.05); other pair-wise differences are given in Table 2.

The soil bacterial community structure was less responsive to management than the fungal community. Although P fertilisation had a significant influence on the profile of bacterial TRFLP’s (P = 0.018; Table 1), the magnitude of the effect was small (R = 0.247; Figure 5A). No shift in the soil bacterial community structure occurred with stocking rate (Figure 5B; P = 0.305, Table 1), however, significant phylogenetic shifts occurred between sampling dates (P<0.05; Table 2). Temporal effects were stronger under different stocking rates than between P fertiliser treatments (Table 2). Across stocking rates, changes in bacterial communities were evident in April, August and September (Table 2; Figure 5B).

The richness of soil fungi increased from d = 5.62 to 6.14 following P fertilisation (P = 0.023), and from d = 6.58 to 7.37 with stocking rate (P<0.001). Fungal richness was affected by sampling date (P<0.05), and was tightly correlated with rainfall (R² = 0.774). There were no interactions between sampling date and pasture management. Bacterial richness was not affected by pasture management treatments and did not display temporal variation (P>0.05).

The capacity for bacterial nitrification (amoA gene abundance) increased significantly with stocking rate and P fertiliser addition (P<0.001; Figures 6A and B), but not with date (Figure 6C), except during the drought occurring during the months of July and October (P = 0.013). The size of nitrogen-fixing bacterial communities (measured by nifH gene abundance) increased with stocking rate (P = 0.027; Figure 7A) but was not affected by P fertilisation (P>0.05; Figure 7B) or sampling time (P>0.05; Figure 8C). Abundance of narG gene copies (nitrate reductase) increased with stocking rate and P fertiliser additions (P<0.05; Figure 8A and B). The abundance of narG varied with sampling date, with lowest relative abundance in July and then increasing from August to November (Figure 8C).

Discussion

Pasture-based agricultural systems are complex and subject to a wide range of management...
practices. These have been aimed at increasing plant production and livestock production, but are increasingly trying to balance sustainability and environmental goals. As soil microorganisms support numerous ecosystem processes underpinning production, sustainability and environmental impacts, there exists potential to manage these processes to achieve further gain. In order to achieve this, knowledge underpinning the responses of soil biological communities and processes to pasture management is needed (Gupta and Ryder, 2003). To gain such knowledge, the technological capacity to reliably measure, quantify and assess management-induced shifts in microbial communities at both community (phylogenetic) and functional (process) levels must be in place. Such technology is now a routine and established component of soil microbiology ecology (e.g., Wakelin et al., 2007) and is moving rapidly into larger capacity formats (Gao et al., 2007; He et al., 2007; Yergeau et al., 2007). In this study, we have demonstrated the direct response of soil microbial communities, including those involved in N transformations, to pasture management.

**Table 2.** Summary of effects (ANOSIM Global $R$ values) of phosphorus fertiliser addition, stocking rate and sampling date on the fungal and bacterial community structures in soil from the Hall site.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Fungi</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphorus</td>
<td>Stocking</td>
</tr>
<tr>
<td>6-Apr-06, 5-May-06</td>
<td>0.59*</td>
<td>0.76*</td>
</tr>
<tr>
<td>6-Apr-06, 5-Jun-06</td>
<td>0.44*</td>
<td>0.91*</td>
</tr>
<tr>
<td>6-Apr-06, 5-Jul-06</td>
<td>0.65*</td>
<td>1.00*</td>
</tr>
<tr>
<td>6-Apr-06, 7-Aug-06</td>
<td>0.54*</td>
<td>0.94*</td>
</tr>
<tr>
<td>6-Apr-06, 5-Sept-06</td>
<td>0.87*</td>
<td>1.00*</td>
</tr>
<tr>
<td>5-May-06, 5-Jun-06</td>
<td>0.15</td>
<td>0.78*</td>
</tr>
<tr>
<td>5-May-06, 5-Jul-06</td>
<td>0.37*</td>
<td>0.926*</td>
</tr>
<tr>
<td>5-May-06, 7-Aug-06</td>
<td>0.59*</td>
<td>0.87*</td>
</tr>
<tr>
<td>5-May-06, 5-Sept-06</td>
<td>0.89*</td>
<td>0.93*</td>
</tr>
<tr>
<td>5-Jun-06, 5-Jul-06</td>
<td>0.41</td>
<td>0.63*</td>
</tr>
<tr>
<td>5-Jun-06, 7-Aug-06</td>
<td>0.26</td>
<td>0.67*</td>
</tr>
<tr>
<td>5-Jun-06, 5-Sept-06</td>
<td>0.83*</td>
<td>0.74*</td>
</tr>
<tr>
<td>5-Jul-06, 7-Aug-06</td>
<td>0.35</td>
<td>0.61*</td>
</tr>
<tr>
<td>5-Jul-06, 5-Sept-06</td>
<td>0.82*</td>
<td>0.69*</td>
</tr>
<tr>
<td>7-Aug-06, 5-Sept-06</td>
<td>0.52</td>
<td>0.52*</td>
</tr>
</tbody>
</table>

*P < 0.05.

**Figure 5.** MDS plot showing resemblance of soil bacterial community structure (T-RFLP) as affected by P fertiliser addition and stocking rates at the Hall field site over the April to September period, 2006.

The application of lime at the Book Book site was the primary management practice affecting microbial communities. Lime has been applied to the soil to mitigate low soil pH; limed field plots had pH$_{CaCl_2}$ of 5.5 whereas non-limed plots had pH$_{CaCl_2}$ of 4.1 (White et al., 2000). In addition to altering the overall composition of soil microbial species (rRNA genotypes), liming also increased richness of the fungal community. Within the limed plots, higher...
primary productivity inputs of organic matter into the soil is likely to have been a key factor increasing the fungal diversity. The addition of lime to soil has been shown to directly affect composition within fungal communities (e.g. Matthies et al., 1997). Furthermore, soil pH is a primary factor affecting microbial species composition and function in soils (Fierer and Jackson, 2006;
Lauber et al., 2008) including those in Australia (Wakelin et al., 2008). The effect of planting with annual versus perennial pasture significantly influenced the structure of the soil bacterial community; however, this effect was secondary to liming. Botanical composition between these treatments varied greatly (White et al., 2000) and this may have resulted in differential selective pressure on the soil bacterial community. Soil bacterial communities have been shown to vary between plant species (Garbeva et al., 2004). However, stock management practices, chemical inputs (e.g. herbicides) and pasture management are also likely to have an impact on the soil microbial community (White et al., 2000). Finally, the differences in botanical composition also affected soil porosity, water relations and associated processes (White et al., 2000), all of which may modify habitat for bacteria.

In addition to altering the community of soil fungi and bacteria, liming also resulted in an increase in the abundance of key functional groups of bacteria involved the geochemical cycling of N (Figure 3). In particular, the increase in abundance of the \textit{nif}/\text{H} and \textit{amoA} genes shows that the biological potential for N$_2$ fixation (N inputs to soil) and nitrification (N cycling within soil) was enhanced with liming. This effect was irrespective of whether the pasture system was annual or perennial. However, White et al. (2000) measured N-fixation in the system in 1996 and found highest rates in the limed, annual pasture treatment. This was attributed to increased clover cover at the time, and this is still potentially an explanatory factor describing N$_2$ fixation in this treatment. Nevertheless, across all treatments a strong correlation ($R^2 = 0.81$) between N$_2$ fixed in 1996 (data in White et al., 2000) and \textit{nif}/\text{H} gene abundance (this data, sampled 2006) suggests a coupling between biological potential and expression of activity within this farming system.

Similarly, the bacterial potential for ammonia oxidation was measured by qPCR detection of a conserved sub-unit of the ammonia mono-oxygenase gene, \textit{amoA} (Rotthauwe et al., 1997). As this step is rate limiting, detection of \textit{amoA} is widely used to measure the biological capacity for the entire nitrification process (De Boer and Kowalchuk, 2001). In grazed pasture systems, nitrification converts N derived from mineralisation of organic matter such as plant debris, fertiliser N or excretal N from animal manure and urine, to nitrate which is suitable for plant uptake or further microbial cycling (Bolan et al., 2004). As such, nitrification is a fundamental component of soil N cycling and fertility. Addition of lime to the soil increased the biological capacity for this process to occur (Figure 3B). Liming-induced increase in \textit{amoA}-gene copies is likely to reflect the increase in productivity and total C and N cycling within this management treatment (White et al., 2000). Increasing soil pH may also support a preferred soil habitat for ammonia oxidising bacteria, however acid-tolerant nitrification can also occur in many soils (De Boer and Kowalchuk, 2001).

Figure 8. (A)–(C) Effects of stocking rate, P fertiliser addition and sampling time on the abundance of the \textit{narG} genes (nitrate reduction) per ng of DNA extracted from Hall field trial soil. Error bars = SEM.
Management practices did not affect the biological potential for nitrate reduction (narG), the first step in the denitrification pathway. Many forms of N losses from pasture systems, particularly leaching/runoff of and in greenhouse gasses, are undesirable due to their potential environmental impact (Bolan et al., 2004). Although the abundance of narG genes in soil cannot predicate the final form of N loss, it may be an indicator of overall potential for the process to occur with inherent implications for fertility and economic loss.

**Hall site**

The three grazing system treatments examined at the Hall site reflect a range in the intensification of grassland farming from low-input management (no fertiliser, low stocking rate) to near-optimal input and utilisation (fertilised and grazed at the higher stocking rate) (Hill et al., 2004) and permitted an analysis of the response of microbial community structure to intensification in grassland use. In addition, the intermediate treatment (fertilised and grazed at the low stocking rate) permits the separate impacts of fertiliser and stocking rate to be explored.

The composition of the soil fungal community was most responsive to pasture management influences, with stocking rate and P fertiliser levels having a similar magnitude of effect (Table 1). Strong shifts in the community structure over time indicated that seasonality also influenced microbial species composition. The implications of management-induced shifts in soil fungal communities on production and ecosystem processes cannot be accurately determined until such changes are characterised. However, different species of soil fungi have varying capacity to cycle fractions of soil organic matter, induce or suppress disease, cycle soil P, or to emit N2O-based greenhouse gases, etc. (Garrett, 1963; Laughlin and Stevens, 2002; Wake lin et al., 2004). As such, species shifts can potentially have large effects on ecosystem function. The richness of soil fungi also increased with intensification of the pasture production system. Increased biodiversity (sensu richness) may provide for a more resilient soil ecosystem (Coleman and Whitman, 2005), able to better withstand and recover from stress and disturbance (Orwin and Wardle, 2004). The increase in richness of soil fungal communities with intensification was in direct contrast to the impact on botanical diversity; unfertilised pastures were invariably more botanically diverse than the fertilised pastures (Hill et al., 2004).

Significant changes in the structure of soil bacterial communities were induced by P fertilisation, but the effect was smaller than that measured in fungal community (Table 1). The soil bacterial community structure was not affected by stocking rate, but did alter between sampling dates. These results show that the bacterial community composition was influenced by pasture management most probably because the management interventions also changed botanical composition and pasture productivity. These results are supported by previous work (e.g. Kowalchuk et al., 2002; Smalla et al., 2001). However, as Kowalchuk et al. (2000) have shown, microbial diversity may not be correlated with botanical diversity in grasslands undergoing secondary succession (i.e. changes induced by management interventions) and the notion that microbial communities will always follow botanical change is likely to be a gross over-simplification of the links between microbial and botanical communities. Application of P in the present grassland system increased pasture production and changed botanical composition. Cover of Bromus spp. (Brome grasses) was increased, that of Vulpia spp. (silvergrass) decreased and clover content was altered (Hill et al., 2004). Both fungal and bacterial community compositions were altered. Stocking rate, however, also changed botanical composition, with tall “competitor” species (P. aquatica and Bromus spp.) becoming dominant in fertilised pasture. T. subterraneum was suppressed when stocking rate was low, whereas T. subterraneum and Bromus spp. were dominant in fertilised pasture when stocking rate was high (Hill et al., 2004). Grazing altered fungal community structure significantly, but did not influence the bacterial community structure. Grazing qualitatively and quantitatively affects C inputs into soil as a consequence of animal waste, trampling and plant root deposition (Kuzjakov et al., 2002; Nguyen, 2003). Alteration in the quality of soil organic matter inputs is likely to be a key factor driving heterotrophic microbial communities. In addition, grazing can affect soil physical properties (e.g. compaction driven decreases in macro-porosity and pore space) which in turn influences habitat for soil microbiota and conditions affecting microbial processes (e.g. O2 limitation, REDOX, water filled pore space, etc.). P fertilisation at the site has altered the botanical composition of the pasture species at the site (Hill et al., 2004).

Effects of seasonality on soil microbial community structure are widely reported (Smalla et al., 2001; Bossio et al., 1998; Kennedy et al., 2005). In many systems, the effects of seasonality are
related to leaf fall (deciduous forests), cold-climate (freezing/snow cover), or plant growth (annual cropping systems). However, in the perennial pasture system investigated here, these factors were unlikely to be significant. Patterns of microbial community structures indicated that the change was gradual over time, despite the fact that soil samples were taken through the opening of the growing season and into an unusually severe spring drought. Based on this sampling, we cannot conclude that the effect is attributable to seasonal difference; a complete year of sampling would be necessary to determine if the biotic community structures under pastures are inherently cyclical. Temporal changes in the quantity and quality of organic matter deposited into rhizosphere soils from plant roots may act as a selective pressure for soil biota, particularly in mixed pastures where growth rates of different plants will vary with temperature and rainfall.

Intensification of the pasture production system (P and grazing) significantly increased pasture growth, clover content and animal productivity, indicating a substantial increase in N₂ fixation had occurred. These changes in pasture management are known to result in long-term increases in the availability of N and C substrates in the soil (e.g. Williams and Donald, 1957). The implications for loss of N from these particular systems (e.g. narG – denitrification) are unknown as the ultimate form(s) of N have not been determined. However, mitigation through altering biological conversion of N to N₂O or other undesirable nitrogenous compounds may be achievable by altering soil structural habitat or by altering the availability of substrates which drive N₂O production. For example, this may be achieved through alteration of grazing regime (altering C:NO₃⁻) or altering pasture species composition; the rooting profiles of different plants affect soil structure, porosity, water infiltration and capture soil N from different profiles of the soil. As Wallenstein et al. (2006) state, environmental factors affecting denitrification, such as O₂, pH, C availability, NO₃⁻ pools, etc., all act through the soil biological community. Understanding impacts of management and environment on functionally important groups of soil biota is the first step to achieve anthropocentrically directed outcomes.

Sampling and methodological considerations

Microbial communities in soils are considered to be highly spatially variable with vast heterogeneity in species diversity. In most studies, DNA extractions for microbiological assessments are made from relatively small samples of soils (e.g. Wakelin et al., 2007). Together, these factors may potentially reduce investigative power to calculate treatment effects within field-based studies. However, as demonstrated in this study, use of careful sampling strategies involving soil sub-sampling minimises spatial variability in microbial communities to levels well below those induced by management treatments.

Assessment of microbial diversity and community structure based on molecular methods inevitably raises questions with regards to DNA extraction efficiency and PCR (primer) amplification bias. Despite these issues being well known, PCR and down-stream tools (particularly DGGE, TRFLP, sequence-based analysis) remain the mainstay of soil microbial ecology research as, despite these limitations, these tools are some of the most useful and powerful available to researchers. However, given that DNA extraction and PCR amplification bias may occur to varying degrees, it is essential to ensure that they occur homogenously across samples (Fromin et al., 2002). These issues have been the subject of rigorous discussion and review previously (e.g. Fromin et al., 2002).

Similarly the validity of using richness and evenness indices to explore microbial community diversity following PCR has received criticism (e.g. Bent et al., 2007). In this work, we have applied Margalefs index to measure richness of bacterial and fungal phylotypes. The context of the study was not to determine total microbial richness per se, but rather to comparatively explore the response of phylotype diversity among treatments; an application considered entirely valid (Danovaro et al., 2007). Recently a suite of new analyses are being applied to provide further insights into the diversity of microbial community following molecular fingerprinting methods (Marzorati et al., 2008). A significant advantage of these tools is the potential to compare aspects of diversity between studies.

Conclusions

Published reports describing the effects of management on soil microbiology in pasture production systems are infrequent. In this work we have shown that the soil microbiota are highly responsive to pasture management practices. In addition to altering the dominant fungi and bacteria present in soil, management also directly influenced groups of organisms involved in key processes underpinning ecosystem productivity and
environmental sustainability. These changes in microbial communities were measurable and quantifiable. However, to gain tangible benefits from managing soil microbial communities and their functions in soils, it will be necessary to further characterise changes and interpret the results in the context of farming systems. Understanding management effects on biological communities involved in soil organic matter cycling will have direct implications on disease incidence, long-term soil fertility, and production system sustainability. However, focus should also be made towards understanding management effects on N-cycling communities; these functional groups are highly responsive to agricultural management and their activities have impacts spanning fertility, production and environmental factors.

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