

Research Paper

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Author for correspondence:

Richard C. Hayes,

E-mail: richard.hayes@dpi.nsw.gov.au

Contrasting soil microbial abundance and diversity on and between pasture drill rows in the third growing season after sowing

Richard C. Hayes^{1,2} , Vadakattu V. S. R. Gupta³ , Guangdi D. Li¹ ,
Mark B. Peoples⁴ , Richard P. Rawnsley²  and Keith G. Pembleton⁵ 

¹NSW Department of Primary Industries, Wagga Wagga Agricultural Institute, Pine Gully Rd, Wagga Wagga, NSW 2650, Australia; ²University of Tasmania, Private Bag 3523, Burnie, TAS 7320, Australia; ³CSIRO Agriculture and Food, PMB 2, Glen Osmond, SA 5064, Australia; ⁴CSIRO Agriculture and Food, GPO Box 1700, Canberra, ACT 2601, Australia and ⁵University of Southern Queensland, School of Sciences and Centre for Sustainable Agricultural Systems, West St, Toowoomba, QLD 4350, Australia

Abstract

Changed spatial configurations at sowing have been investigated as a strategy to minimize interspecific competition and improve the establishment and persistence of multi-species plantings in pastures, but the impact of this practice on the soil microbiome has received almost no previous research attention. Differences in populations of bacteria and fungi in the surface 10 cm of soil in the third year following pasture establishment were quantified using quantitative polymerase chain reaction and terminal restriction fragment length polymorphism methods. Populations were compared on, and between, drill rows sown to either the perennial grass phalaris (*Phalaris aquatica* L.), perennial legume lucerne (alfalfa; *Medicago sativa* L.) or the annual legume subterranean clover (*Trifolium subterraneum* L.). Results showed that soil microbial abundance and diversity were related to plant distribution across the field at the time of sampling and to soil chemical parameters including total carbon (C), mineral nitrogen (N), pH, and available phosphorus (P), potassium (K) and sulfur (S). Despite the 27-month lag since sowing, pasture species remained concentrated around the original drill row with very little colonization of the inter-row area. The abundance and diversity of bacterial and fungal populations were consistently greater under drill rows associated with higher total C concentrations in the surface soil compared with the inter-row areas. Our results showed that the pH and available nutrients were similar between the subterranean clover drill row and the inter-row, suggesting that soil microbial populations were not impacted directly by these soil fertility parameters, but rather were related to the presence or absence of plants. The abundance of bacteria and fungi were numerically lower under phalaris rows compared to rows sown to legumes. The richness and diversity of fungal populations were lowest between rows where lucerne was planted. Possible explanations for this observation include a lower C:N ratio of lucerne roots and/or a lack of fibrous roots at the soil surface compared to the other species, illustrating the influence of contrasting plant types on the soil microflora community. This study highlights the enduring legacy of the drill row on the spatial distribution of plants well into the pasture phase of a cropping rotation and discusses the opportunity to enhance the microbiome of cropping soils on a large scale during the pasture phase by increasing plant distribution across the landscape.

Introduction

Soil health and function are of key interest to farmers and their advisors who are seeking to improve the utilization of soil resources and to increase the resilience of production systems in the face of extreme and variable climatic events. Soil microbial communities are fundamental to soil health and function (Doran and Zieff, 2000; Van Der Heijden *et al.*, 2008; Toju *et al.*, 2018) and it is therefore important to identify and implement management strategies that enhance the abundance and diversity of desirable microbial communities. Some of the recognized beneficial influences of microorganisms on plant growth and nutrition include the rhizobium-legume symbiosis for biological nitrogen fixation, mycorrhizal associations for phosphorus (P) uptake, disease suppression and improved soil structure (Gupta *et al.*, 2019b).

However, beyond these examples the dynamic relationships between plant and microbial communities in agricultural landscapes remains relatively poorly understood. It is clear that plant growth promotes microbial proliferation by providing a source of nourishment, e.g., carbon (C) as the energy source, and it is known that different plant species provide soil habitat conditions [C and nitrogen (N) concentrations] that are conducive to the proliferation of specific microbial communities (Osborne *et al.*, 2010; Thakur *et al.*, 2015; Tiemann *et al.*, 2015;

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Gupta *et al.*, 2019b). Therefore, plant diversity in grassland ecosystems has been speculated to increase soil microbial diversity and promote biological functions; especially in soils lower in organic matter, as high plant diversity may provide a greater supply and quality of resources to microorganisms (Johnson *et al.*, 2003; Loranger-Merciris *et al.*, 2006; Eisenhauer *et al.*, 2013). It is also known that particular microbial populations are advantageous for plant communities by facilitating organic matter decomposition, nutrient cycling and suppression of root diseases (Gupta *et al.*, 2019b). Plant species present in grassland systems such as lucerne, subterranean clover and phalaris are generally associated with microorganisms involved in symbiotic (legume-rhizobia) and non-symbiotic (grass-diazotrophic) N fixation and mycorrhizal associations. Microorganisms vary in terms of their response to varying quantities and types of biologically available C, other energy sources or nutrient substrates. For example, as per the currently accepted ecological classification scheme for soil bacteria, some taxa are more associated with greater labile C pools (copiotroph) such as in rhizosphere soil, whereas the taxa that dominate in soils with lower organic C availability are known as oligotrophic. It has been suggested that bacteria belonging to β - and γ -Proteobacteria and Bacteroidetes are considered copiotrophic whereas genera belonging to Acidobacteria are considered oligotrophic, that is, low in C turnover and high in C-sequestration potential (Fierer *et al.*, 2007; Trivedi *et al.*, 2016). Thus, changes in the balance between C turnover and retention through variation in microbial communities due to plant rhizosphere effects may be crucial because of its effects on soil fertility, structure and sustainable crop production.

Agricultural practices have large and often unintended impacts on soil microbial communities. Manipulation of the pasture drill row may be a convenient way to positively influence soil microbial communities and function in integrated crop/forage production systems as the drill row can impact large areas at a fine scale with relatively little cost and inconvenience to the farmer (Hayes *et al.*, 2017a). The creation of the drill row is typically preceded by significant disturbance to flora through tillage and/or applications of agri-chemicals. Ecologically, it is presumed that this disturbance would revert the soil microbiome to an early stage of succession (Crews *et al.*, 2016) after which it would progress along the successional gradient for as long as the management of that field permitted. In phased pasture-crop rotations common across south-eastern Australia, a 4–7 yr phase of continuous annual winter crop production is commonly broken by a 3–5 yr pasture phase that includes perennial and self-regenerating annual forage species (Kirkegaard *et al.*, 2011). The pasture phase represents an opportunity for succession in the microbiome due to the low level of disturbance that typically occurs. However, no study that we could find has previously examined the impact of phased pastures on the microbiome or explored the extent to which the pasture drill row might impact the microbiome.

This study is a first step in understanding the legacy of the initial pasture drill row and its impact on microbial abundance and diversity in the rhizosphere. It asks the question whether differences in soil biota attributable to the drill row can be observed in the third year after sowing and tests two hypotheses: (i) that the abundance and diversity of microbial populations varies according to whether a grass or a legume was initially sown in the drill row, and (ii) that the abundance and diversity of microbial populations is greater in the soil directly beneath the original drill row compared to the area between drill rows.

Materials and methods

A series of experiments were established to test the impact of alternative spatial configurations of mixed pasture swards on pasture productivity and persistence (Hayes *et al.*, 2017b). A subset of treatments from one experiment in that series was sampled to determine the extent and nature of differences in soil microbiology attributable to different pasture drill rows and is the focus of this paper.

Site and treatment details

The experiment was conducted at a field site near Wagga Wagga, NSW, Australia. The soil was classified as a red Kandosol (Isbell, 1996) and is described in greater detail by Conyers *et al.* (2018). Pastures were direct-drilled with a three-point linkage seeder fitted with narrow points and press wheels set at 250 mm spacings on May 30, 2012. The seeder was fitted with two distributing cones and eight sowing tynes and was modified in such a way that seeds of two pasture species could be delivered to alternate tynes from different cones. Plots were $6 \times 4 \text{ m}^2$, replicated three times. Approximately 150 kg ha^{-1} of Starter 15 fertilizer (14.7% N, 13% P, 12% S; Incitec Pivot Fertilizers, Melbourne) was broadcast across the experimental area immediately prior to sowing. Weeds were controlled during the 6 months prior to sowing using glyphosate as required.

Treatments sampled for soil microbiology included subterranean clover (*Trifolium subterraneum* L.) sown in every drill row (SC-only), subterranean clover and phalaris (harding grass; *Phalaris aquatica* L.) sown in alternate drill rows (Phal:SC) and subterranean clover and lucerne (alfalfa; *Medicago sativa* L.) also sown in alternate drill rows (Luc:SC). Cultivars and sowing rates of subterranean clover were identical in each treatment and included a mixture of three cultivars, Riverina, Coolamon and Bindoon, sown in equal proportions by weight at a total of 4 kg ha^{-1} of viable seed. That is, where subterranean clover was restricted to only half the drill rows, the number of seeds of that species placed in the designated drill rows was doubled compared to where subterranean clover was sown in every drill row in order to keep the weight of subterranean clover seed sown constant across treatments. Two cultivars of lucerne, Aurora and Genesis, were sown in equal proportions by weight at a total of 3 kg ha^{-1} of viable seed. Phalaris cv. Sirolan was also sown at 3 kg ha^{-1} . Legume seeds were inoculated with the commercial strain of rhizobia for lucerne (RRI 128) and subterranean clover (WSM 1325) by applying a peat media the day prior to sowing.

Plant frequency

The physical location of the sown species relative to the original drill row was quantified on July 14, 2014 with a quadrat with external dimensions of $0.5 \text{ m} \times 0.5 \text{ m}$, divided into 100 cells each $50 \text{ mm} \times 50 \text{ mm}$. The quadrat was placed parallel to the visible drill rows on a representative area of the plot after the herbage was clipped to a height of approximately 20 mm. The number of squares containing the base of a respective sown species was counted and calculated over the total quadrat as 'frequency (%)' (Lodge and Gleeson, 1984).

Soil sampling

Soil samples were taken twice on September 17, 2014, first for soil microbial abundance and composition, and secondly for soil chemical characteristics. For the first soil sampling, ten cores

were taken from each sampling location within each plot, either on or between the original pasture drill rows, using a foot-corer with 20 mm diameter sampling to 100 mm depth. As the initial pasture experiment was sown on a row spacing of 250 mm, samples taken from between the drill rows were approximately 125 mm from either original drill row. Samples were kept cool and transported immediately back to the laboratory, where roots were separated and discarded. The remaining soil was refrigerated at 4°C and shipped in an insulated storage box to the analytical laboratory for molecular (bacterial and fungal composition) analysis.

DNA extraction and quantification

DNA was extracted from 0.3 g (dry weight equivalent) samples of soil, in duplicate, using the PowerSoil DNA kit (www.qiagen.com) following the manufacturer's protocol. Mechanical disruption of the soil was accomplished by bead-beating using Fastprep (2×) (speed 5.5, 30 s; FP120; Q-biogene Inc., CA, USA) after which the final DNA extracts were eluted using 100 µL of warmed (60°C) C6, for 5 min, to maximize DNA yield, and the extracts were stored at -80°C. DNA extracts were also further cleaned using the MinElute 96 UF PCR Purification Kit (www.qiagen.com.au) and DNA eluted into nuclease free water.

All DNA samples were tested for purity using a NanoDrop ND1000 (Thermo Fischer Sci. Inc.) and the concentration of DNA against a DNA standard (λ -phage DNA; $R^2 = 0.98$) using the QuantiT PicoGreen dsDNA assay (Invitrogen, MA, USA). The final extracted DNA was diluted tenfold in molecular grade H₂O and 3 µL was used per 15 µL polymerase chain reaction (PCR) reactions. Abundances of total bacteria and fungi were quantified using group-specific primers that have been used extensively for analyzing soil bacterial communities and shown not to amplify mitochondrial/chloroplast DNA [bacteria: F968/R1378 (AACGCGAAGAACCTTAC/CGGTGTGTACAAGGCCCGGGAACG) (Smalla *et al.*, 2007) and fungi: FR1/FF390 (TTGGTCATTAGAGGAAGTAA/TTYGCTGYGTTCTTCATCG) (Vainio and Hantula, 2000)] based on reagents in the QuantiTect SYBR Green PCR kit (Qiagen, Vic., Australia). All PCR reactions were carried out on a Stratagene Mxpro3000P qPCR system (Agilent, Vic., Australia). Quantitative-PCR (qPCR) was performed against standards. Standard curves for bacterial qPCR were generated using a pGEM-Teasy vector-based clone and the number of 16S rRNA gene copies was estimated, whereas for fungal qPCR, known amounts of culture DNA from *Rhizoctonia*, *Fusarium* and a *Trichoderma* isolate were combined to make a standard curve for the quantification of fungal DNA in the samples. Details of PCR conditions are described by Gupta *et al.* (2019a).

Analysis of microbial diversity

Bacterial and fungal community compositions were analyzed using the terminal restriction fragment length polymorphism (TRFLP) method. Bacterial TRFLP was conducted using universal primers 8F and 1520R; the forward primer was labeled with FAM and the reverse primer with HEX. PCR cycles consisted of 5 min at 95°C followed by 30 cycles of 30 s at 94°C, 60 s at 55°C and 120 s at 72°C, with a final extension of 10 min at 72°C. Analysis of fungal diversity using TRFLP was carried out using primers ITS 1F and ITS 4 (Gardes and Bruns, 1993), labeled with FAM and HEX, respectively. PCR cycles consisted of 5 min at 95°C followed by 35 cycles of 60 s at 94°C, 60 s at 56°C and 90 s at 72°C, with a

final extension of 10 min at 72°C. Bacterial gDNA controls included *Escherichia coli* and *Listeria* (non-pathogenic species) as positive controls.

Amplified DNA fragments for both bacteria and fungi were cleaned using the SureClean kit (Bioline Inc.) and DNA was re-suspended in 20 µL sterile water. Cleaned PCR product (8 µL) was then digested using restriction enzymes AluI and CfoI for 3 h at 37°C, and the reaction was stopped by heating to 65°C for 20 min.

Fungal community DNA was amplified from 14 ng of template DNA using the ITS1F.FAM forward and ITS4R.HEX reverse primers (Gardes and Bruns, 1993). PCR was carried out in a 35 µL total volume using 0.4 µM of primers, 0.2 mM of dNTPs, 1× PCR buffer (Qiagen, Australia), and 4 units of HotStarTaq DNA Polymerase (Qiagen, Australia). The reaction conditions were 94°C for 1 min (denaturation); 56°C, 1 min (annealing); 72°C, 1.5 min (extension) for 35 cycles using a thermocycler. In addition to the sample DNA, a negative control without DNA and positive controls with *Rhizoctonia*, *Saccharomyces* species and *Fusarium* culture DNA were included in every PCR run.

Terminal restriction fragments were separated using capillary separation (ABI 3730 DNA analyzer, Australian Genome Research Facility, Adelaide) with a LIZ500-250 size marker (ABI). Size and intensity data were interpreted using GeneMarker analysis software (SoftGenetics Inc.), with a minimum cut-off of 200 intensity units used to distinguish terminal restriction fragments from background noise.

Soil chemical analyses

Two soil coring tubes, each 25 mm in diameter, were inserted at each respective sampling location. Results from only the two surface depths, 0–5 and 5–10 cm, are reported here as they relate directly to the sampling for soil microbiology. Soils were air-dried in a fan forced dehydrating oven set at 40°C and passed through a 2 mm sieve. Coarse organic material and gravel too large to pass through the sieve were discarded from the sample. Soil pH was determined on a 1:5 soil:1 M KCl solution shaken for 1 h and measured at 20°C. The same extractant was used for the determination of mineral N (ammonia [NH₄⁺] plus nitrate [NO₃⁻]) concentration using a QuickChem® 8000 series flow injection analysis system. Total concentrations of soil C and N were determined by dry combustion (Leco, 1995) according to method 6B2b of Rayment and Lyons (2011). Colwell phosphorus and potassium (Colwell, 1963) as well as available sulfur (KCl-40; Blair *et al.*, 1991) were determined at a commercial laboratory (Incitec Pivot, Werribee).

Statistical analysis

Data collected from the subset of treatments reported in this paper were analyzed with an analysis of variance (ANOVA) using a complete randomized block model in Genstat (v18.1.0, VSN International Ltd.) with sampling location as the fixed term and replicate as the random term for each soil depth.

All the TRF data were analyzed for size and intensity using GeneMarker analysis software (SoftGenetics Inc.), using default settings for TRFLP analysis with a minimum threshold of 200 intensity units used to distinguish true TRFs from background noise. Band intensities (heights) were imported into Microsoft Excel and the relative abundance of a TRF in a TRFLP profile was calculated against total peak heights of all TRFs in the profile. All peaks with heights that were <0.5% of the total peak height were not included in further analyses.

Multivariate statistical analysis for community composition comparison and diversity indices calculation from TRFLP fragment data was performed using PRIMER-E (Primer 7; Clarke and Gorley, 2006). TRF abundance data were Hellinger transformed and Bray–Curtis dissimilarity matrices (+1) were constructed, statistical analyses performed, and diversity estimates calculated. Cluster analysis was performed with the Similarity Profile analysis (SIMPROF) test (Clarke *et al.*, 2008). Significant differences in community structure were tested for location, plant type and interaction models with permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) and analysis of similarity (ANOSIM; Clarke and Ainsworth, 1993). Canonical analysis on principle co-ordinates was performed for factor groups that were found significant with PERMANOVA only. For significant PERMANOVA results, the nature of the location-based differences and their relationships to soil physico-chemical properties (as predictor variables) were tested using distance-based linear model (distLM) analyses (Clarke and Gorley, 2006). For this, all specified factors were used to identify variables that explained significant ($P < 0.05$) amounts of variation in bacterial and fungal community structures. Heatmaps for relative abundances of TRFs were generated using Minitab vR2018a (Minitab Inc., PA, USA). Shannon diversity (H), Pielou's evenness (J), Margalef's richness (d), the number of individuals (N) and relative abundances of bacteria and fungi were analyzed with ANOVA using Genstat (v18.1.0, VSN International Ltd.).

Results

Pasture biomass and distribution of sown species

Pasture botanical composition and biomass for the experimental site was previously reported in Hayes *et al.* (2017b). Pasture biomass increased from 0.7–2.0 Mg DM ha⁻¹ in year 1 to 9.0–10.7 Mg DM ha⁻¹ in the third growing season after sowing. The Phal:SC sward was consistently more productive ($P < 0.05$) than the Luc:SC and SC-only swards in all but the final year where Phal:SC and SC-only swards had similar quantities of biomass. The proportion of unsown weeds was greatest in the subterranean clover sward compared to other treatments and although incrementally increasing with time in all treatments, never exceeded 16% in any treatment (Hayes *et al.*, 2017b).

In year 3, the distribution of all sown species remained concentrated close to the respective drill row established 2 years prior. The basal frequency (%) for subterranean clover in the SC-only treatment was calculated as the average of both drill rows sampled and was higher in the inter-row area compared with both perennial species (Table 1).

Soil chemical characteristics

There were significant ($P < 0.05$) differences in total C, mineral N and available phosphorus (P), potassium (K) and sulfur (S) between sampling locations in the surface 5 cm of soil (Table 2). The values of all parameters were generally lower in the inter-row area compared to those on the original pasture drill row. However, soil mineral N and available S and K were significantly lower on the subterranean clover drill rows compared with those planted to the perennial species, and statistically similar to the inter-row area. There was no significant treatment effect on soil pH in the surface 5 cm, but in the 5–10 cm depth either under subterranean clover drill rows or in the inter-row area

Table 1. Basal frequency (%; \pm standard error) of subterranean clover, phalaris or lucerne in winter of year 3 on the original drill rows compared to the inter-row area (approximately 125 mm from the original drill row)

Species	Drill row	Inter-row	Sample number
Lucerne	60 \pm 40	0	$n = 3$
Phalaris	50 \pm 10	23 \pm 8	$n = 3$
Subterranean clover	77 \pm 19	40 \pm 14	$n = 6$

between subterranean clover drill rows soil pH was generally lower compared to other sampling locations. Available K was also significantly higher under the phalaris and lucerne drill rows compared to other locations ($P < 0.05$), although differences were not as large as in the surface 50 mm (Table 2).

Soil microbiology

Soil bacteria were more abundant under pasture drill rows that were established over 2 years prior to sampling, compared to soil collected in the inter-row area. The abundance of bacteria under the phalaris drill row was less than that under either the lucerne or subterranean clover rows and was intermediate between the drill rows planted to legumes and areas between drill rows (Fig. 1A). Similarly, the abundance of soil fungi was greater under previous pasture drill rows compared to the inter-row area. It was again intermediate under the phalaris rows compared to most other drill rows and the inter-row area (Fig. 1B).

Bacterial species richness (Fig. 2A) and diversity (Fig. 2B) was generally greater on the original drill row compared to the inter-row area for both the phalaris and subterranean clover. However, no difference was found in species richness and diversity between the lucerne drill row and the corresponding inter-row area (Fig. 2A and B), despite the large observed change in bacterial abundance (Fig. 1A). Fungal species richness and diversity were also generally higher on the drill row compared to the inter-row area (Fig. 2C and D), although differences between treatments were not significant ($P = 0.08$) due to large error terms. Values for both parameters were substantially lower in the inter-row area of the Luc:SC treatment compared with all other locations.

Multivariate analysis of TRF data with edaphic variables as vectors clearly showed the vectors for the dissimilarity between on-row and inter-row areas. The proportion of each factor in explaining variation in bacterial and fungal community structure due to sampling location was explored with distLM analysis. For bacterial community structure, organic C, C:N ratio, S, NO₃⁻ and NH₄⁺ were the significant ($P < 0.001$) variables (Fig. 3A), whereas for the fungal community structure mineral N, C:N ratio, NH₄, S and K were the significant ($P < 0.05$) predictors (Fig. 3B). Total N and Colwell P had little impact on the composition of bacterial and fungal communities. Canonical analysis of principle ordination of relative abundances for TRFs, constrained by location and edaphic factors, showed significant variation in microbial community composition between on-row and between-row samples.

Results from heat maps depicted distinct fungal and bacterial communities in soil (Fig. 4). There were clear differences between samples taken from the drill row compared to those from the inter-row area for both groups of organisms. There was generally good agreement in samples taken from the subterranean clover drill rows across the different experimental treatments. Two distinct groups of bacteria were apparent, one more dominant on

Table 2. Total carbon (C), mineral nitrogen (N), pH, available phosphorus (P), potassium (K) and sulfur (S) in the 0–5 and 5–10 cm layers at different sampling locations in September 2014, relative to the pasture drill row established in May 2012

Sampling location	pH _{KCl}	Total C (%)	Mineral N (kg ha ⁻¹)	P–Colwell (mg kg ⁻¹)	K–Colwell (mg kg ⁻¹)	S–KCl40 (mg kg ⁻¹)
0–50 mm depth						
B/n Luc:Sub	5.49	1.90	4.5	33.7	413	4.7
B/n Phal:sub	5.29	1.88	3.4	38.0	423	5.2
B/n Sub	5.36	2.02	5.2	48.0	397	4.9
Luc row	5.80	2.63	15.5	39.0	660	10.5
Phal row	5.51	2.37	10.1	38.3	797	13.0
Sub row	4.86	2.35	8.8	29.8	359	7.3
<i>P</i> -value	0.097	<0.001	0.02	0.017	<0.001	<0.001
<i>l.s.d.</i> _{0.05}	–	0.266	4.46	9.39	75.4	2.76
50–100 mm depth						
B/n Luc:Sub	4.22	1.26	3.4	28.0	330	7.6
B/n Phal:sub	4.16	1.26	2.7	16.7	300	7.5
B/n Sub	3.92	1.21	2.5	43.3	320	8.3
Luc row	4.29	1.28	4.4	24.7	390	7.7
Phal row	4.19	1.26	3.0	29.3	383	6.6
Sub row	3.98	1.24	2.9	26.4	293	5.6
<i>P</i> -value	0.037	0.987	0.233	0.136	0.023	0.03
<i>l.s.d.</i> _{0.05}	0.229	–	–	–	61.4	1.77

B/n, between (i.e., the inter-row areas); Sub, subterranean clover; Luc, lucerne; Phal, phalaris.

the inter-row area while the other was more dominant on the drill row. The drill rows also conferred a greater diversity of fungal communities compared to the inter-row area, and the fungal communities sampled from the drill row appeared to differ according to the pasture species that were sown.

Discussion

The presence of a growing plant is known to make a significant impact on soil microbial communities both in terms of their abundances, composition and activity utilizing the enhanced supply of C and nutrients from root exudation and turnover (Kuz'yakov and Razavi, 2019). This single point-in-time study illustrates an unanticipated legacy of pasture drill rows established more than 2 years prior to sampling soil microbial populations. Despite the 27-month lag, pasture species remained concentrated around the original drill row where the majority of the root influence would be concentrated, undoubtedly driving results we observed in the microbiome as the microbial abundance and diversity was reduced in the inter-row area where plant frequency was lower.

High plant frequency in the drill row was associated with increased total C and increased mineral N in the surface 50 mm. The chemistry of surface soil in the subterranean clover drill row contrasted that of soil in the rows sown to the perennial species, phalaris and lucerne, by being lower in pH ($P = 0.09$) and lower in the available macronutrients P, K and S ($P < 0.01$). These differences were not reflected in the abundance of bacteria and fungi, which tended to be lower under the phalaris rows compared to either the lucerne or subterranean clover rows. This result suggests that microbial abundance and diversity are being driven more by the composition of the plant material which is

scarcely reflected in the standard soil chemistry analysis. Previous studies have documented changes in decomposition and mineralization rates between these contrasting species following the termination of the pasture phase (Bolger *et al.*, 2003; Angus *et al.*, 2006). However, few studies have examined the microbiome under living swards and we can therefore only speculate that factors such as C:N ratios, root architecture, root turnover or the presence of more recalcitrant components in the tissue, such as lignin, impacts the microbiome. The current study supports the first hypothesis that the abundance and diversity of microbial populations vary depending on whether a grass or a legume was initially sown in the drill row, which is consistent with previous reports of changes in the composition of microbial communities induced by the presence of N₂-fixing legumes (Osborne *et al.*, 2010). These observations highlight the importance of retaining pasture legumes in production systems for the benefit of soil microflora.

Fungal species richness and diversity were substantially lower between rows in the lucerne/subterranean clover treatment compared to most other sampling locations ($P < 0.05$). We postulate that this may be the result of lower fibrous root proliferation in the inter-row area at the soil surface, on account of the strongly tap-rooted lucerne being sown in every second drill row. Lucerne is the perennial legume species most commonly grown in phased rotations with crops in dryland environments across south-eastern Australia (Dear *et al.*, 2010; Kirkegaard *et al.*, 2011) largely due to its superior drought tolerance and capacity for more consistent inputs of biologically fixed N₂ than annual legumes across a range of environments (Peoples *et al.*, 2012). Despite these benefits, lucerne swards in this region are characteristically low in ground cover (Hayes *et al.*, 2018) due to the high

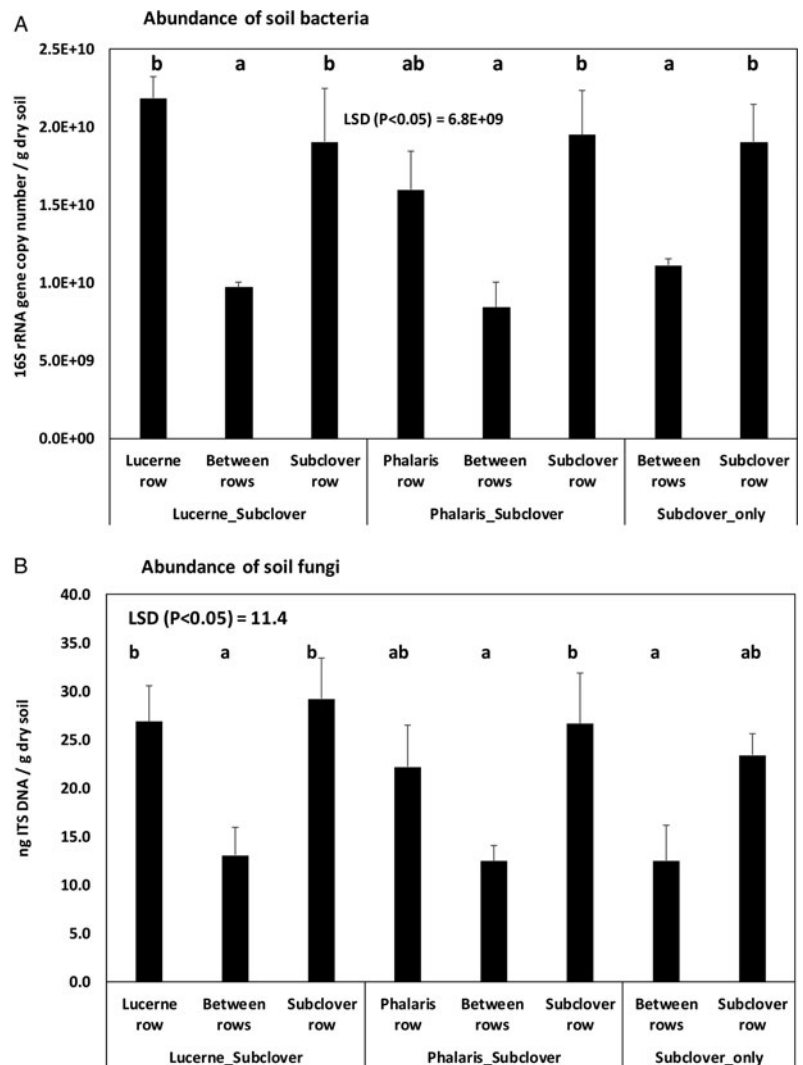


Fig. 1. Localized spatial variation between the pasture drill row and the inter-row area in the abundance of soil bacteria (A) and fungi (B) in pure subterranean clover swards (SC-only) and swards sown to subterranean clover and either phalaris or lucerne in alternate drill rows (adapted from Hayes *et al.*, 2017a). Bars marked with the same letter are not significantly different ($P > 0.05$).

degree of interspecific competition with subterranean clover (Dear and Cocks, 1997) and its superior capacity to exploit water in the soil profile to a much greater depth (Ridley *et al.*, 2001; Sandral *et al.*, 2006; Hayes *et al.*, 2010). The competitiveness of lucerne was a primary motivation for planting the two species in separate drill rows. Further research is required to determine the extent to which increased microbial diversity and abundance can be achieved in lucerne swards through a greater and more even distribution of fibrous-rooted species among the lucerne crowns and in particular, between drill rows.

There is little evidence that soil nutrition, within the range of values reported in this study, confers a direct impact on soil bacteria and fungi abundance and diversity. Rather, soil microbiology appears to be impacted primarily by the presence or absence of plants, as observed in the clear contrast in the microbiome under the pasture drill row compared to the sparsely populated inter-row area. It is noted that most of the responses in soil chemistry were plant-driven responses since fertilizer, for example, was not applied to the drill row and so changes in P, K and S are an indirect result of plant activity (see Table 2). We suggest that most of the differences in soil microbiology observed in the current study were also indirect and plant-driven.

The enduring legacy of the pasture drill row on the microbiome is an unanticipated but important finding. One might

have presumed that in the third year of a self-regenerating pasture, remnants of the original drill row would be scarce due to more uniform plant coverage across the field, especially for a self-regenerating annual species such as subterranean clover. On the contrary, present results demonstrated an enduring legacy in terms of plant distribution, which has resulted in significant shifts in soil chemistry and biology. It is possible that this result is due to the relatively low soil organic matter at this particular experimental site which potentially makes relatively subtle differences in the inter-row compared to the drill row more distinguishable. If we accept that a more resilient soil is one with a richer and more diverse microbiome, the implication of this research is that land managers need to find methods to increase the plant frequency across a field, especially during a pasture phase that is intended as a period of soil recovery in an otherwise intensive annual cropping program (Kirkegaard *et al.*, 2011; Angus *et al.*, 2015). Thus, the findings of this study suggest that greater diversity of plant species with contrasting habits, such as legume species vs non-legume species, as well as tap-rooted vs fibrous-rooted species, could positively influence the microbiome. In a recent study combining global observations with an experimental microcosm study, Delgado-Baquerizo *et al.* (2020) observed that soil biodiversity is significantly and positively associated with multiple ecosystem functions hence critical for maintaining soil

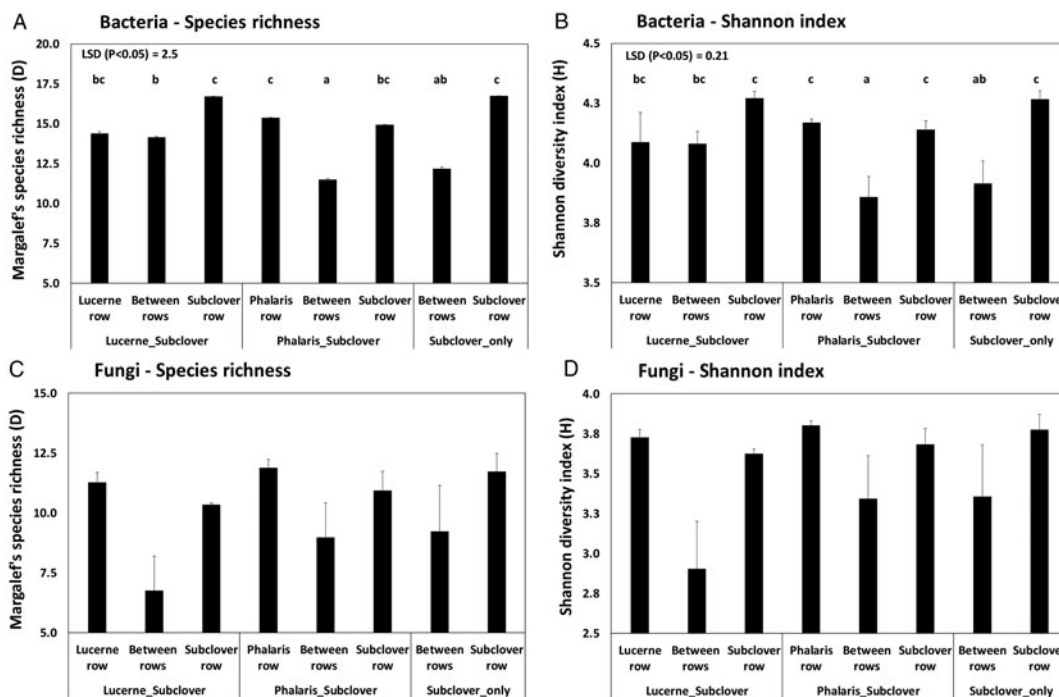


Fig. 2. Indices of species richness (Margalef; A and C) and diversity (Shannon; B and D) for soil bacteria (A and B) and fungi (C and D) sampled from the surface 100 mm in the third year after the establishment of drill rows sown to either subterranean clover, phalaris or lucerne, compared to soil sampled from the inter-row area. Bars marked with the same letter are not significantly different ($P > 0.05$); no bars marked with letters indicate that no significant differences exist between treatments ($P > 0.05$).

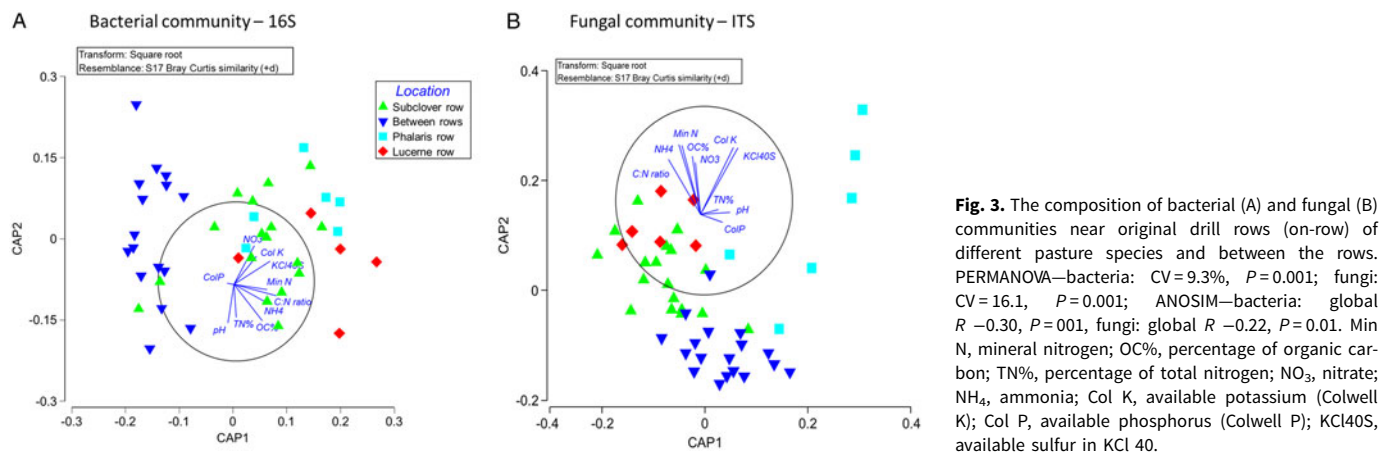


Fig. 3. The composition of bacterial (A) and fungal (B) communities near original drill rows (on-row) of different pasture species and between the rows. PERMANOVA—bacteria: CV = 9.3%, $P = 0.001$; fungi: CV = 16.1, $P = 0.001$; ANOSIM—bacteria: global $R = -0.30$, $P = 0.01$; fungi: global $R = -0.22$, $P = 0.01$. Min N, mineral nitrogen; OC%, percentage of organic carbon; TN%, percentage of total nitrogen; NO₃, nitrate; NH₄, ammonia; Col K, available potassium (Colwell K); Col P, available phosphorus (Colwell P); KCl40S, available sulfur in KCl 40.

functionality locally and across biomes. Increasing plant frequency in semi-arid environments such as those across southern Australia would likely need to be achieved without increasing plant density, as dense pasture stands are commonly not sustainable in such water-limiting environments. McCormick *et al.* (2014) quoted densities of 6–20 plants m^{-2} as being adequate in a perennial species such as lucerne to fully utilize available resources (especially water), while Bowman *et al.* (2002) suggested that only 8 plants m^{-2} might be required to contribute adequate amounts of fixed N to support subsequent cropping phase in semi-arid environments of south-eastern Australia. Such low densities present obvious challenges to increasing the frequency of plants across the landscape in order to facilitate a more abundant and diverse microbiome and speaks to the importance of

self-regenerating annual species to fill the gaps in such environments at times of the year when resources are more abundant.

Increasing plant frequency in the pasture phase to promote greater abundance and diversity of soil microorganisms across the cropping landscape will likely involve adopting narrower row spacings. Crop species grown at narrower row spacings generally achieve higher yields due to better utilization of available resources and less weed competition (Scott *et al.*, 2013). On the other hand, farmers wishing to reduce tillage frequency and stubble burning are forced to compromise and move toward wider row spacings to manage stubble loads. The benefits of minimum tillage cropping practices often outweigh grain yield reductions (Scott *et al.*, 2013). The same challenges exist when farmers are looking to establish a pasture in the year following a crop as the

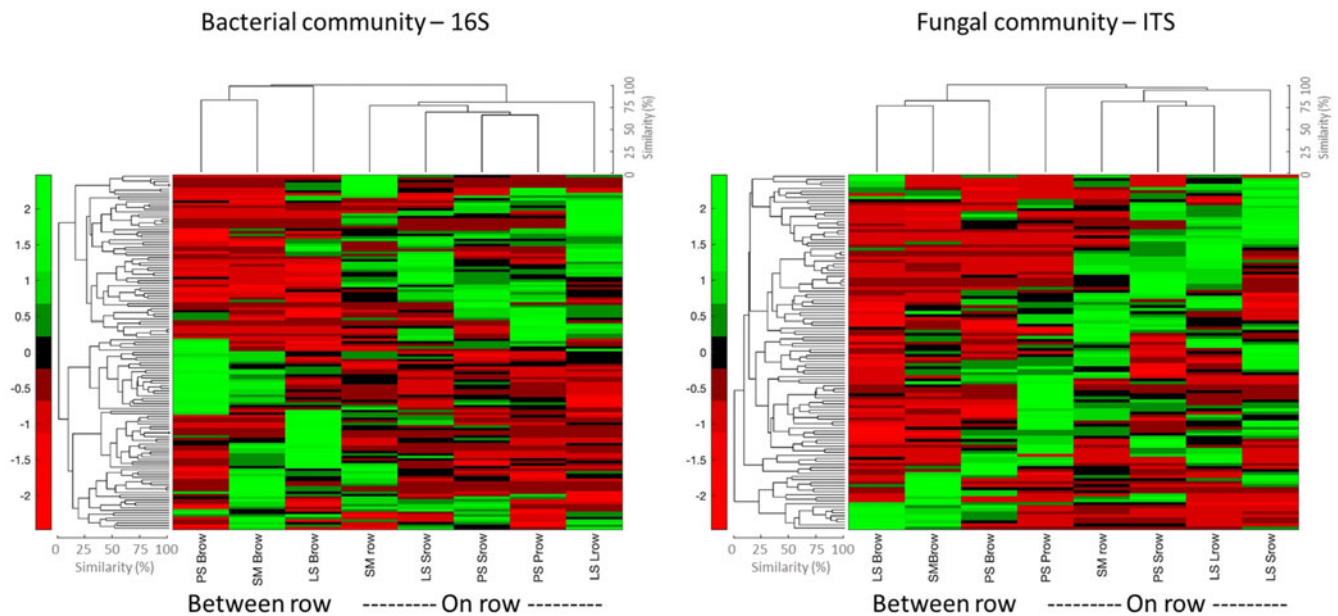


Fig. 4. Heat maps for the abundances of TRFs representing different soil bacterial (A) and fungal (B) communities as measured using TRFLP analysis. The color variation is presented for each TRF across different treatments (horizontal). Green color represents most abundant and red color represents least abundant. Horizontal cluster diagram represents percent similarity variation in the community composition between the different treatments. Vertical cluster diagram represents percent similarity variation between TRF groups. PS, phalaris-subterranean clover sward; SM, pure subterranean clover; LS, lucerne-subterranean clover sward; Srow, subterranean clover drill row; Prow, Phalaris drill row; Lrow, Lucerne drill row, Brow, between drill rows.

residual stubble burden will often not permit seeding equipment affixed with tynes at narrow spacings. However, on the basis of the enduring legacy of the pasture drill row that has been identified in this study, we postulate that yield reductions are potentially compounded over the life of the pasture, and the resilience of the cropping landscape could well be reduced, due to the greater inter-row area that we now know harbors a constrained microbiome.

Conclusion

The soil microbiome is fundamental to soil function and resilience. Increasing the abundance and diversity of microbial populations would seem to be a key strategy to maximize soil function and resilience. An obvious priority for future research would be to quantify the effects of the observed spatial heterogeneity in abundance and diversity of bacteria and fungi in terms of detailed phylogenetic and functional microbiome composition (using metagenomic techniques) and actual functions. Our study indicated that increasing plant frequency is the factor that most affects the abundance and diversity of both bacterial and fungal populations. Paying close attention to practices at establishment that would increase plant frequency offers great promise to significantly enhance the microbiome on a large scale across the cropping landscape. Achieving a higher plant frequency would seem to be particularly important in phased pastures where benefits compound over the life of the stand, and where legacy effects of the pasture phase have now been shown to be enduring.

The current study raises many additional questions that will require further research to better understand the potential impact of improving soil biology through increased plant frequency during the pasture phase. These include: (a) Can economic benefits of increased microbial diversity and abundance be quantified, perhaps through nutrient cycling or pest/disease management? (b) Are there environments or seasons in which increased microbial

diversity and abundance result in higher plant yields or persistence? (c) What other agronomic practices (e.g., changed crop rotations and multi-directional plantings) or mechanical modifications (e.g., scatter plates on seeders) might enable greater plant frequency to be achieved in short-duration pasture phases? In light of the unanticipated findings of the current study, we suggest that the microbiome in the pasture phase deserves greater research attention in order to increase the resilience of pasture/crop rotations.

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