Metabolites and biological entities in Victorian dairy farm soils and their relationships to management

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Abstract
Up to 60% of phosphorus (P) in soil is organic. Because plants generally access organic P *via* biochemical transformation to orthophosphate (inorganic P) (Marschner et al., 2011), understanding how organic P is converted to inorganic P may ultimately allow for reductions in orthophosphate fertiliser use. Three analytical techniques to better understand organic P processes were applied to dairy farm soils from treatments within P × K fertiliser trials at three sites in Victoria, Australia: the Northern Irrigation Region (NIR), south-western Victoria (SW Vic), and Gippsland. Analyses included: (i) physicochemical to measure total and available N, P and K, and pH, etc.; (ii) microbial community analyses to determine what microbes are in the soil and what is their function; and (iii) metabolomics to study the metabolites that microbes, plants and animals produce and use within the soil. Physicochemically, the sites were found to be significantly different ($P < 0.05$) for most soil analytical measures. P and K fertiliser applications increased available P and K. About 15 µg DNA/g soil was extracted from the Gippsland and SW Vic soils, compared to only ~9 µg DNA/g from the NIR soil. Archaea sequences (9.7 million) were more readily identified from the soil samples versus bacterial or fungi sequences (3.5 and 3.0 million respectively). Metabolomic analyses found approximately 1800 potential metabolites. Sixty metabolites, from a target list of ~200 compounds were identified including 24 amino acids, 16 sugars (including *myo*-inositol) and 6 organic P compounds. Cluster analyses of bacterial, archaeal and metabolomic data differentiated the sites. While the microbes and metabolites were closely related in concentration and identity at the NIR and SW Vic sites, the Gippsland soils were clustered due to differences in fertiliser treatments.

Keywords
Dairy soils, organic compounds, microbes, phosphorus

Introduction

Soils are a complex matrix of interacting geochemical (e.g. clays, water) and biological (e.g. microbes, plants) entities which affect how any metabolite is formed, transformed and transferred through the matrix (Daniel, 2005). Geochemical interactions occur within the soil due to soil pH, temperature and texture, among others (Kröger et al., 2013; Bronick and Lal, 2005). Biological interactions by microbes, plants, fungi and animals (e.g. earthworms) are the cause of many biochemical transformations of chemical compounds such as sugars (Gougoulias et al., 2014)), organic (Vance, 2001) and inorganic N (Masclaux-Daubresse et al., 2010), and phosphorus (P) (Nash et al., 2014). A greater understanding of these transformations may help to find ways to improve pasture quality and/or quantity (Pereira e Silva et al., 2013) by developing methods to access nutrients locked up in the soil matrix (Stutter et al., 2012).

As Australia's third largest rural industry, dairy is a significant contributor to the Australian economy (Dairy Australia, 2015a). Victoria has approximately 68% of Australia's dairy farms (Dairy Australia, 2015b), and there is an interest to improving the productivity of dairy farms through an understanding of the plant and soil interactions (Gourley et al., 2015). In particular, interest in how plants utilise N, P and other trace elements such as K, is ongoing.

This research is part of a larger project into the effects of P and K fertiliser on pasture production on dairy farms in the three largest dairy regions in Victoria. P is an important nutrient in plants, and its continued application to originally P deficient Australian soils, has resulted in relatively large pools of organic

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P in dairy soils (Nash et al., 2014). By combining traditional agricultural analyses (e.g. Olsen P) with metabolomics techniques (the study of metabolites) and microbial community analyses, we are investigating the biochemical transformations occurring at the molecular and microbial scale. The research project focuses on organic P, although other metabolites were also investigated so as to understand the transformations occurring in the soil matrix.

Methods

Location

The three main dairy areas of Victoria are in Gippsland, Northern Irrigation District (NIR) and South West Victoria (Figure 1). The present study was based on a subset of treatments within three P × K trials (Table 1) established for the Sustaining Productive Dairy Soils (SPDS) project (Aarons, unpublished data).

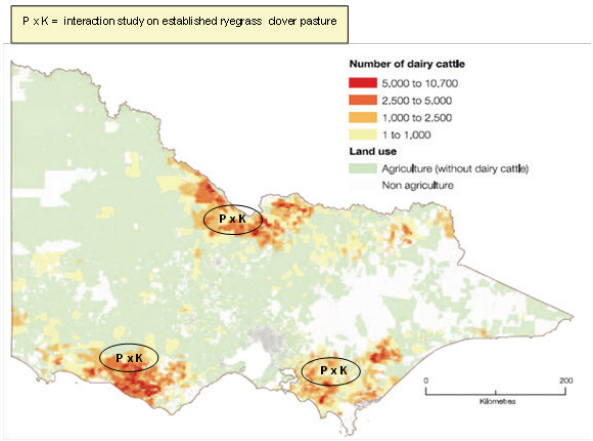

Figure 1. The location of the main dairy regions in Victoria, Australia where the three SPDS P × K trials are located.

Table 1. P and K fertiliser treatments in the SPDS trials, expressed as relative rates (cf. agronomic maintenance rate) of fertiliser applied per year. The 0× and 2× P maintenance rate treatments were sampled to maximize chance of discerning a P fertiliser effect.

		P maintenance rates				
		0	**0.25**	**0.5**	**1**	**2**
K maintenance rates	**0**	2	*1*	*2*	*1*	2
	0.25	1	*1*	*1*	*1*	1
	0.5	2	*1*	*2*	*1*	2
	1	1	*1*	*1*	*1*	1
	2	2	*1*	*2*	*1*	2

Sample collection

Soil samples (0-10 cm depth, c. 500 g composited from c. 40 individual cores) were collected from treatment plots in September 2014. Samples were immediately placed on ice and transported to the laboratory. Subsamples for metabolomics and soil community analyses were kept at -80C. Subsamples for physicochemical analyses were dried at 40C.

Analyses

Physicochemical analyses included pH, EC, Total P, Available P, Total C, Total N, Extractable S, ammonium (NH_4-N), nitrate, and exchangeable cations using standard methods. Metabolomic analyses used liquid-chromatography mass spectrometry (LC-MS) to search for all metabolites extracted with 80% acetonitrile. Data identifying retention time, mass/charge and concentration were collected in an untargeted manner. This data set was interrogated with a target list of compounds examined in-house and via the Maven database (Clasquin et al., 2002). Total community microbial DNA was extracted from the samples and analysed using microbial community sequencing. Taxomic libraries were created by targeting DNA sequences specific to bacterial, fungal and archaeal communities. Sequences obtained from the samples were clustered into operational taxomic units (OTU) and the identity of microbe OTUs was determined using the Ribosomal Database Project (Cole et al., 2014).

Results and Discussion

Physicochemical analyses

All physicochemical analyses showed significant differences between the farms ($P < 0.05$). For example, average Olsen P for the Gippsland (34 mg/kg) and SW Vic (29 mg/kg) samples was almost double that of NIR (17 mg/kg) samples (Table 2). Total P was more equivocal, with NIR having an average 412 mg/kg while for SW Vic and NIR it was 383 and 323 mg/kg, respectively.

Regardless of site, increasing P from 0× to 2× P fertiliser maintenance treatments increased Olsen and Colwell P ($P < 0.05$), and Total P by up to 55 mg/kg ($P = 0.053$). Addition of K fertilizer to plots reduced analyte concentrations between 2 and 10 fold. These effects were strongest for P (0.01 M $CaCl_2$) and Ammonium for SW Vic and NIR plots, while Gippsland plots to K were more varied but smaller. However, significant effects between K maintenance levels were only found for soil K, increasing from an average of 0.27 to 0.38 meq/100 g for plots with 2 × K.

Table 2. Selected P physicochemical measurements of the sampled sites.

		Olsen P (mg/kg)		Colwell P (mg/kg)		Colwell P Digest (mg/kg)		Total P (mg/kg)	
P level		0	2	0	2	0	2	0	2
Gippsland	Min	17	23	28	37	110	110	260	300
	Max	44	67	88	160	170	230	520	650
NIR	Min	10	19	23	45	50	76	360	380
	Max	18	28	41	68	82	110	440	460
SW Vic	Min	19	32	47	77	120	170	190	110
	Max	28	43	68	100	160	200	370	470

*Phosphorus fertiliser maintenance level applied annually.

Metabolomic analyses

Approximately 1800 untargeted features (metabolites and their adducts) were found using the untargeted metabolomics analyses. Using multivariate analytical techniques such as principle component analyses (PCA), it was found that the metabolites clustered into the three dairy regions studied (Figure 2). For SW Vic and NIR, metabolites clustered more tightly than those from Gippsland samples. 60 metabolites were identified including 24 amino acids, 16 sugars, 6 organic P compounds and 3 nucleotides. Of note, myo-inositol, the backbone of the largest organic component in soil, phytate, was identified. On average, amino acids were an order of magnitude more abundant cf. sugars while nucleotides and organic P compounds were an order of magnitude less. NIR and SW Vic samples only had 76% and 58% of the amino acid and nucleotide content of the Gippsland samples, respectively.

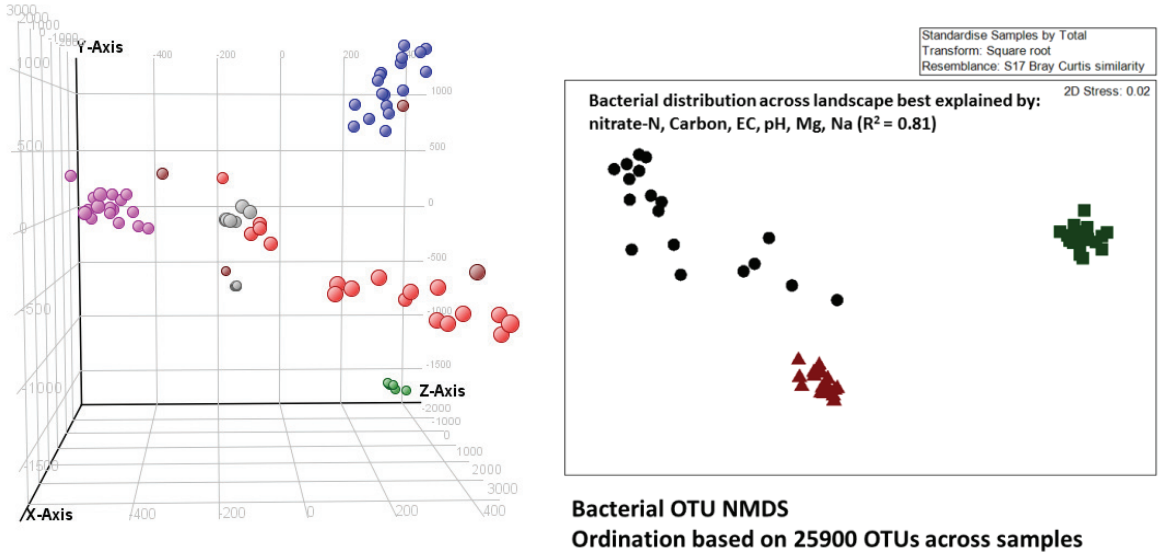

Figure 2. Left: PCA of untargeted data of metabolites found in soil samples from dairy farms in Gippsland (red), NIR (blue) and SW Vic (pink). Pooled Biological Quality Controls (grey); test of samples with no processing (brown); and target standards at higher concentrations (green) also shown. Right: Non-metric multidimensional scaling (NMDS) of bacterial operational taxonomic units (OTUs) taken from samples from Gippsland (circle), NIR (square) and SW Vic (triangle) sites.

Soil microbial community analyses

Similarly to the metabolomics analyses, soil microbial community data for bacteria and archaea were clustered by location, but the treatments for the Gippsland samples lead to a more varied concentration and

type of microbe compared with the NIR and SW Vic farms (Figure 2). This similarity of clustering using two different techniques suggests, for Gippsland soils at least, that the metabolomic and soil biology data are linked, as in the metabolites are predominantly sourced from microbes rather than the metabolites being from pasture plants, cows or soil geochemistry. For bacteria, more than 80% of the clustering was attributed to effects of soil pH, and nitrate, carbon, magnesium and sodium concentrations. Clustering of archaea OTUs was attributed to the same factors (R^2 = 89%). Fungal OTUs showed overlap of SW Vic and Gippsland samples, with a separation between those and NIR samples (R^2 = 66%). Across all sites, the largest groups of microbes identified included proteobacteria, acidobacteria, actinobacteria and bacteroidetes.

Conclusion

The soils from each site were physicochemically, microbially and metabolically very different. PCA and NMDS of the metabolomic and microbial data shows a similar pattern of a gradient of metabolites and microbes concentrations across the Gippsland site, while within each NIR and SW Vic site, metabolites and microbes varied little, regardless of N, P or K treatment. Further analyses is being undertaken to identify the linkages that lead to similar results for different targets and the methods used to identify them. For instance, fungi of the order Glomerales significantly increased (P = 0.026) if plots had been fertilised (vs. control plots). Similarly, seven metabolites were found to significantly vary in concentration depending on the rate of K fertiliser application. These metabolites included those involved in polysaccharide formation (D-glucuronolactone), vitamin transformations (D-Galactono-1,4-lactone, 4-aminobutanoate and aspartic acid) or related to other sugars found in plants. This research highlights how different advanced analytical techniques such as metabolomics and microbial community analyses may reveal how and why soil processes are occurring.

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