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Characterising the soil ecosystem phenotype associated with relatively low nitrate-N concentrations

Steve Wakelin[a,*], Paul Maclean[b], Vanessa Cave[b], Jizhong Zhou[c], Gwen-Aëlle Grelet[d], David Whitehead[d]

[a] Scion, PO Box 29237, Christchurch 8440, New Zealand
[b] AgResearch Ltd, Lincoln Science Centre, Private Bag 4749, Christchurch, New Zealand
[c] Institute for Environmental Genomics, University of Oklahoma, Norman, OK, USA
[d] Manaaki Whenua - Landcare Research, PO Box 69040, Lincoln, New Zealand

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ABSTRACT

In agricultural systems, elevated concentrations of soil nitrate (NO_3^--N) pose an environmental risk. Nitrate is highly mobile, and can be transported through soil into aquatic ecosystems resulting in eutrophication. Furthermore, under anaerobic conditions nitrate-N can undergo microbial denitrification. This latter process is a significant source of N_2O, a potent greenhouse gas. The goal of this research is to determine (1) if there are soils, from under live-stock grazed (pastoral) land use, that contain lower than expected (anomalous) nitrate-N per total N content, and (2) characterise the phenotypes of these soils based on physicochemical and microbial attributes. Using a set of 50 soils that have been fully characterised based on edaphic properties, and for which GeoChip environmental functional microarray data is available, the ratio of nitrate-N to total N, an expected value was determined for the 50 soils. Using a 'leave one out' prediction strategy, soils anomalously high ($n = 13$) or low ($n = 14$) in nitrate-N were determined. The phenotypes of these two groups were characterised based on the edaphic and molecular (functional gene and associated phylogenetic association) data. Significant (permutation $p < 0.05$) variation in mid-level carbon (C) and nitrogen (N) cycling gene categories were evident between groups of soil with relatively low or high nitrate N levels. Low nitrate-N soils were characterised by increased abundance of genes involved in reductive acetyl CoA pathway, acetyl-CoA carboxylase, glyoxylate cycling, starch and chitin degradation, and nitrogen reduction (denitrification). Furthermore, the increased occurrence of these metabolic pathways was associated with increased abundance of Classes of Firmicute, Actinobacteria, and some groups of Proteobacteria and fungal taxa. In the group of soils with anomalously low nitrate-N, changes in the function and composition of the microbial community occurred with increased ratios of C:P, C:N, and C:S., and lower sulphate-S contents. These results indicate that a general widening of the stoichiometry of C with other nutrients maybe an important driver, or associative factor, linked with changes in the microbial community function. Opportunities to shift soil ecosystems to a low nitrate-N phenotype may be best achieved through alteration of C inputs to soil or changing C-cycling ecophysiology. This maybe achieved through selection of plants for specific (quantitative and qualitative) rhizosphere C allocation profiles.

1. Introduction

In natural soil ecosystems, the cycling of carbon (C), comprising the primary source of energy for most taxa, is closely coupled to the cycling of other major and minor elements (Kirkby et al., 2011). The balance between mineralization and immobilisation of nutrients such as phosphorus (P), sulphur (S), and nitrogen (N) can thereby affect wider ecosystem productivity, and constitute an important force shaping the above ground ecosystems, particularly through selection of plants with different functional traits (e.g. mycorrhizal associations for P acquisition, or root nodule N-fixing) (Wardle et al., 2004).

Addition of exogenous nutrients into soil ecosystems, either from organic or inorganic sources, can disrupt the extent of coupling between elemental cycles (Wakelin et al., 2013a). This is most often observed in intensified agricultural systems that often receive mineral or organic fertiliser additions. However, the nutrient enrichment of agroecosystems can be costly to the environment (Robertson and Vitousek, 2009), and such trade-offs need to be weighed against the

* Corresponding author.
E-mail address: Steve.A.Wakelin@scionresearch.com (S. Wakelin).

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economic return associated with greater production. Of particular importance is formation of high concentrations of mobile N in soils, either from mineral fertiliser addition or animal urine patches. In particular, nitrate-N (NO_3^-) is highly soluble and elevated concentrations pose a risk for movement off-farm into groundwater or other aquatic ecosystems (Schlesinger, 2009). Furthermore, given suitable physiochemical conditions, nitrate-N can be denitrified by soil microorganisms into gaseous N_2O, which has a strong radiative forcing potential and contributes to global climate change and ozone depletion (Davidson, 2009; Erisman et al., 2011).

Soil microbial ecosystems are massively complex in both taxa present and biogeochemical processes they support (Giller, 1996; Curtis et al., 2002; Bardgett and van der Putten, 2014). The wider ecosystem phenotype, at any point in time, is an emergent property that integrates changes in biophysical conditions, from predatory-prey relationships, trophic interactions, moisture status, nutrient inputs etc. (Schmidt et al., 2011). As such, given the complexity of biophysical interactions that underpin biogeochemistry in soil, it is not surprising that direct links between individual functional genes (i.e. those associated with a biochemical transformation step) are often weak, unclear, or not evident. For example, a significant amount of research has investigated the microbiological basis of N cycling, particularly the molecular ecology of nitrogen-cycling bacteria and archaea (e.g. Hallin et al., 2009; Lindsay et al., 2010; Tao et al., 2017). While these investigations have greatly enhanced our understanding of the taxa involved in soil N transformations, and the ecology of these organisms across a range of agroecosystems, opportunities to translate this knowledge into management options to reduce loss of nitrogenous compounds to on-farm, while not incurring significant production penalties, have been difficult to realise (Cassman et al., 2002; Coskun et al., 2017). A possible reason may be related to the single-nutrient focus of much research to date. For example, investigations of soil N biogeochemistry typically focus on the ecology of one or more N-cycling (functional) genes in relation to the occurrence of a range of N forms over time, among different management systems etc. (e.g. references in Henry et al., 2006; Kandeler et al., 2006; Philippot et al., 2007; Hallin et al., 2009). Oftentimes, these do not consider the importance of the role of coupling to other elemental cycles, nor the importance of trace elements in regulating microbial nutrient cycling processes (Perakis et al., 2006; Wurzburger et al., 2012; Jean et al., 2013).

The wider goal of the research programme that this work contributes to is 'the identification of opportunities to reduce soil nitrate accumulation by management of on-farm carbon inputs'. Within this programme, the aim of this work was to assess the physiological properties of the microbial ecosystem linked to the occurrence of soil phenotypes either anomalously high or low in nitrate-N. Given that many metabolic pathways affect nitrate formation or loss, and the tight coupling of N with C-cycling, the approach used a high-density environmental DNA microarray analysis (GeoChip; Yan et al., 2015) across 50 well-characterised pasture soils (Wakelin et al., 2013b). The specific aims were to: (1) identify soils with anomalously high or low nitrate N given variation in other soil properties, (2) identify changes in soil N or C-cycling functional genes that may explain differences in nitrate-anomalous soils, and (3) characterise soil physicochemical properties associated with changes in functional genes, and thereby identify opportunities to manage outcomes of ecosystem functions.

2. Methods and materials

2.1. Soils and their physicochemical properties

The base soil data originates from the New Zealand 50 pastures project (Wakelin et al., 2013b). The original dataset comprises a broad characterisation of soils based on 41 edaphic and environmental properties, spanning total and extractable concentrations of major and minor nutrients, metals, pH, electrical conductivity, cation exchange capacity, bulk density, annual rainfall, evapotranspiration, and so forth. The soils were collected from 10 geographic zones, spanning 11 NZ soil groups (Hewitt, 1998); correlations of these soil groups to the USDA classification systems (Soil Survey Staff, 2010) are given in Supplementary Table 1. While all samples were collected from under permanent pasture, these spanned high- through to low-intensity farming systems; operationally we grouped these as being from 'dairy' or 'other' (e.g. dry stock grazing) based farming systems, respectively, and this land-use grouping is supported by increased concentrations of elements such as P and N within the dairy-based systems (Wakelin et al., 2013b).

Soils were collected from a single location at each site (point sampling), such that variation among the dataset can be validly assessed but not within-field variation; indeed, this was not the intention of the study. Each soil sample was screened through a 2 mm diameter mesh to remove coarse plant material, stones, and other debris. Enough soil was processed to provide at least 2 kg of screened soil from each point-location. This was sealed in plastic bags and kept at 4 °C. Sample of this soil was used for physicochemical analysis and extraction of DNA; these extractions were conducted within 5 days of soil collection (Wakelin et al., 2013b).

The base dataset contains a number of C and N measurements important to this study: total C, total N, nitrate-N, and anaerobically mineralisable N (AMN). In addition to these, hot water extractable organic C (HWOC), a property often closely associated with the soil organic matter content (Ghani et al., 2003), and aromaticity of the HWOC (as a measure of lability) was determined on the soils. HWOC was determined using the general process described by Ghani et al., (2003). Briefly, 3 g (dry wt. equivalent) of field-fresh was initially extracted with 30 ml of water at 20 °C for 30 min on tumble-shaker. The soils were recovered by centrifugation at 3500 rpm and decanting off the supernatant. The soil extraction was repeated with another 30 ml di. water, but at 80 °C for 16 h. After extraction, the supernatant was collected, filtered to 0.45 µm, and analysed by a Shimadzu 5000A TOC analyser. Aliquots of the HWOC fraction were standardized to 45 µg ml^{-1} and the aromatic component quantified by UV absorbance at 254 nm (FLUOstar Omega microplate reader, BMG Labtech; Volk et al., 2002). An increase in the aromatic content of the (A_{254}) was inferred to indicate the quality of the soil organic matter (HWOC) in terms of availability for microbial decomposition, where greater aromatic/condensed C content (phenols etc) maybe related to chemical recalcitrance to decomposition (Volk et al., 2002).

For all variables, residual plots were used to check for evidence of departures from the assumptions of normality and constant variance. There was no strong evidence to support a requirement for data transformation.

2.2. Soil functional genomics; GeoChip environmental microarray

From a sub-sample of each of the soils, DNA had been extracted and stored at −80 °C as described by Wakelin et al. (2013b). Thus, the microbial community in each soil can be directly compared with the edaphic and environmental properties associated with each sample, and in relation to wider farm system intensification etc. In order to assess the functional capacity of the soil ecosystems to cycle C and N compounds, the analysis of the DNA samples using the environmental microarray GeoChip 5.0 (He et al., 2007; Yan et al., 2015) was conducted. The microarray system has probe sets covering > 150,000 genes and spanning 410 gene categories from nutrient cycling to antibiotic resistance. As such, it can be used to provide a comprehensive view for potential ecosystem function (e.g. Wakelin et al., 2013a; Yan et al., 2015). A full description of the workflow for GeoChip analysis, including data pre-processing for these soils, is given in Wakelin et al. (2016). Given the low DNA concentrations obtained from soils 2, 18, 29, 33, and 35, these samples were processed using whole community genome amplification (WCGA) using the phi29 enzyme system (Wu et al., 2006). This resulted in significant bias in the resultant GeoChip

Table 1
Summary of variation in soil C and N variables among two a priori defined farming systems; high-input dairy grazing and others.

Grazing system	Total C	HWOC[a]	Aromaticity[b]	Total N	AMN[c]	Nitrate-N	C:N
Dairy[d]	4.65 ± 0.48	1334 ± 109	0.6533 ± 0.048	0.4529 ± 0.045	141.8 ± 17.81	71.05 ± 8.43	10.04 ± 0.285
Other[e]	4.22 ± 0.31	1377 ± 92	0.5459 ± 0.025	0.4255 ± 0.030	144.1 ± 14.31	40.26 ± 4.76	9.872 ± 0.177
p[‡‡]	0.171	0.939	0.019	0.191	0.764	0.046	0.161

Values provided are the mean ± standard error of the mean (SEM).
[a] Hot water extractable organic carbon.
[b] Absorbance at 254 nm of the HWOC component of the soil C.
[c] Anaerobically mineralizable N.
[d] Dairy = farming systems used to support dairy grazing; typically high input of fertilisers etc; $n = 21$.
[e] Other = farming systems for non-dairy based livestock grazing (sheep etc); $n = 29$.
[‡‡] p-Value associated with the two-tailed, unpaired t-test.

results for these samples (Wakelin et al., 2016). As such, these five sample sets were excluded from this analysis in this study.

Data from the remaining 45 samples were sorted into gene families, filtered by biogeochemical function, and the information associated with C and N cycling genes extracted. This resulted in a final dataset containing over 18,000 probes covering 35 gene subcategories (spanning hemicellulose cycling to nitrogen fixation), and 169 genes (e.g. *nir*K type denitrification). By aggregating data from probe to gene, then from gene to subcategory, hierarchical analysis is possible that provides different levels of resolution.

2.3. Experimental testing

2.3.1. Soil carbon and nitrogen content: variation with intensification and underlying soil and environmental properties

Variation in the carbon and nitrogen content of soils was analysed between the two levels of land-use intensification (dairy farming systems versus others). Variables tested included total C, HWOC, C-aromaticity, total N, nitrate-N, ANM, and the C:N ratio. Testing was conducted using unpaired, two-tailed t-tests.

Associations between the C and N-associated variables, to the wider variables across all sites were independently tested using simple linear regression. The purpose of this latter analysis was to investigate underlying relationships that might not be directly associated with land-use intensification, such as climate or base-soil variables. Similarity in regression coefficients (R-squared values) to soil variables for each of the C and N samples were determined. Distances among variables were calculated using the index of association, and between samples using Bray-Curtis distances as described in Clarke and Gorley (2015).

2.3.2. Identifying soils with anomalous nitrate-N concentrations and relationships with physicochemical properties

Analysis was conducted to determine if sets of soils with anomalously low or high nitrate-N contents could be established. As nitrate-N varied with land-use intensification, the analysis was based on the relationship between $\mathbf{NO_3}^-$ and soil total N content. Anomalous soils were identified using a 'leave-out-one' prediction strategy in which each soil was individually omitted from the dataset, its $\mathbf{NO_3}^-$-N content predicted based on a simple linear regression on soil total N content, and its standardized residual evaluated. The standardized residuals were then ranked to identify soils with anomalously low and high nitrate-N contents. For subsequent analysis, soils were classified in the lower nitrate-N than expected group if the standardized residual was less than -0.6, and in the higher than expected group if it was > 0.6.

Individual simple linear regressions of the standardized residuals on the other physiochemical properties were then used to explore possible reasons why the $\mathbf{NO_3}^-$-N content differed from that expected based on the total N content alone. The residual means square error (RMSE), adjusted R^2, and the p-value for the fitted model were used to compare the amount of variation explained by the individual physiochemical properties.

2.3.3. Variation in function gene and taxa abundances between soils with higher and lower than expected nitrate-N

For GeoChip signal intensity data, each gene, gene family, or gene category was calculated as average within the 'relatively high or low nitrate N' soil groups. This was determined relative to all samples (i.e. not just between high and low nitrate N soil groups), ensuring results are conservative outcomes. For each gene, the log-ratio was calculated of the normalised intensities for each gene to the average normalised intensity for the entire sample. To aid interpretation, log-ratio data was transformed into log fold-change statistics which, unlike log-ratios, are linear in response and symmetric about zero. Testing for effects was done using single-factor permutation ANOVA in the lmPerm package in R (R Core Team, 2015; Wheeler et al., 2016).

Similarly, taxa information was extracted from the NCBI accession numbers associated linked to each GeoChip probe set. Phylogenetic information was retrieved at family level and differences in taxa contributing C and N cycling genes functions in the relatively low and high nitrate N soils tested using permutational ANOVA (as above).

3. Results

3.1. Soil carbon and nitrogen content: variation with farming system intensification and underlying soil and environmental properties

Total C and HWOC contents of soils were similar between high (dairy) and low (other) intensity farming systems (p values of 0.440 and 0.765, respectively; Table 1). However, the aromaticity of the HWOC component was higher in soils under dairy-based land use (Fig. 1A; $p = 0.036$).

Similarly, total N, AMN, and the C:N ratio were all similar across the two levels of system intensification ($p > 0.05$; Table 1). However the concentration of nitrate-N was 77% higher in soils collected from under dairy-grazed systems ($p = 0.001$; Fig. 1B). In soils used for dairy grazing, average nitrate–N concentrations were 71.05 mg kg^{-1} soil, whereas in soils collected from other grazing systems, the average was 40.26 mg kg^{-1} soil (Table 1).

A wide number of correlations existed between soil and environmental variables and the concentrations of different forms of C and N in the soils ($p < 0.05$). These are summarised in Fig. 2. Importantly, there was virtually no overlap in the group of variables correlated with C-aromaticity and other samples, while variables associated with soil total C, total N, and HWOC strongly overlapped (Fig. 2). However, HWOC had some strongly correlated (Persons r) variables: total Cd ($r = 0.395$), moisture at time of sampling (Θd; $r = 0.432$), and anion storage capacity (ASC; $r = 0.544$). While the nitrate-N content was associated with a number of variables common to total C, total N, and HWOC, it also had a number of unique variables: AMN, elevation, total N, volume weight, CEC, total C, and extractable organic S (Fig. 2).

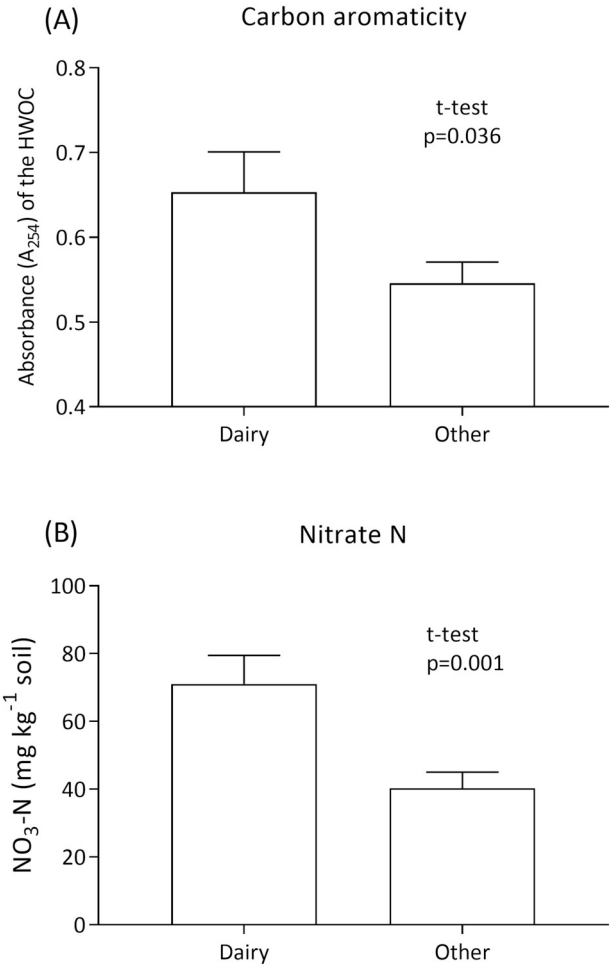

Fig. 1. Average of (A) the aromaticity of hot water extractable organic carbon (HWOC), and (B) nitrate-N content in soils under dairy-grazing (n = 21) or other (e.g. sheep grazing; n = 29) pastoral land uses. Data presented as mean + SEM.

3.2. Identifying soils with anomalous nitrate-N concentrations and relationships with physicochemical properties

Soils were identified in which nitrate-N levels were higher or lower than expected based on the total N content (Suppl. Fig. 1). Soils (n = 13) with anomalously high nitrate-N were identified: soils 19, 15, 40, 21, 20, 24, 43, 37, 9, 13, 10, 30 and 8 (in rank order of increasing anomaly; i.e. the absolute standardized residual is increasing). Of these, 10 were from under dairy-based (high-intensity) grazing systems, and three from under low-intensity grazing. Soils with anomalously low nitrate-N (n = 14) were: 39, 7, 36, 50, 42, 31, 34, 48, 41, 26, 44, 47, 46, and 11 (in rank order of decreasing anomaly). Among these, 10 were from low intensity and four from under high intensity land-use systems. Hereafter, we refer to these as groups of relatively high or relatively low nitrate-N soils.

The results of testing (simple linear regressions) of relationships between the standardized residuals and soil physiochemical properties are presented in Supplementary Table 2. These were conducted on the entire 50 soils dataset. Of the 39 physiochemical properties, eight explain a statistically significant ($p < 0.05$) amount of variation in the standardized residuals. Electrical conductivity (E.C.) explains the most variation (33.2%), followed by eFe (20.9), C/P ratio (19.2%), AMN/total N ratio (13.5%), C/N ratio (13.4%), elevation (12.5%), eAl (10.2%), and anion storage capacity (ASC) (7.8%). Note that the prefix 'e' refers to $CaCl_2$-extractable fractions of each element/nutrient. The linear relationship with the standardized residual is positive for E.C., eFe, and the AMN/total N ratio, indicating that soils with higher than expected nitrate-N contents tend to have higher levels of these physiochemical properties (and vice versa for soils with lower than expected nitrate-N). Conversely, for C/P ratio, C/N ratio, elevation, eAl, and ASC, the relationship is negative, suggesting soils with higher than expected nitrate-N contents tend to have lower levels of these physiochemical properties.

Differences in soil and environmental properties between the 14 relatively low nitrate N soils, and 13 high nitrate N soils were assessed using permutational ANOVA; the summary results are given in Table 2. The most significant effects (p-value) were associated with nitrate-N and E.C.; there is a very strong association between these variables (Pearson's $r = 0.804$; $p < 0.0001$) across the pasture soil dataset. Other than nitrate-N, the only other nutrient that directly differed between the two soil groups was sulphate-S (greater concentration in the high nitrate soil; $p < 0.05$). Other 'nutrient' associated differences were associated with ratios of N with C, P with C, and the AMN/total N ratio.

3.3. Variation in functional gene abundances between soils with higher and lower than expected nitrate-N

Total cobalt was highly correlated with several genes and taxa (> 0.7) but weakly with nitrogen levels (−0.13, for total nitrogen, −0.06 for nitrate-N). In preliminary data-testing, this effect was found to dominate both correlation and canonical correlation analyses (not shown) involving both the physical and GeoChip data, while reducing the separation of these on soils grouped as relatively high and low in nitrate. To more fully explore the interaction between the abundances of functional genes and the physical datasets, cobalt was removed and the analyses repeated. Furthermore, as total and extractable Co (eCo) were highly correlated ($r = 0.88$), eCo was also removed from the analyses.

At the gene-family level, carbon degradation and carbon fixation genes, and denitrification genes were significantly ($p < 0.05$) greater lower in low nitrate soils than high nitrate soils (i.e. were less negative). In contrast, chitin synthesis genes (family = 'major C biomolecule' within GeoChip ontology), were in much greater abundance in the high N soils (Table 3).

At mid-level gene grouping, seven categories were significantly (permutation $p < 0.05$) varied in log fold-change abundance among the relatively high and low nitrate-N soils. These are summarised in Fig. 3. The abundances of chitin, denitrification, glyoxylate cycle, reductively acetyl CoA pathway, and starch degradation genes were greater in low nitrate-N soils.

Analysis of variation in individual genes found significant differences in abundances (fold change) of 24 genes between the relatively high and low nitrate soils (Fig. 4). Within the C-fixation pathway, six genes associated with 3- or 4-hydroxybutyrate cycles (fumarase 3HP4HB, C Co hydratase DiC4HB, AACT DiC4HB, suc CoA red DiC4HB, and mch) were associated with low nitrate N soils. In addition, Calvin cycle genes (Rubisco Glau Rhiz Cryp; a rubisco large subunit gene from a photosynthetic algae), CsoSCA (carboxysomal beta-like carbonic anhydrase), and two acetyl-CoA associated genes (pcc = propionyl-CoA carboxylase, and fthfs = formyl tetrahydrofolate synthetase involved in reductive acetogenesis). Within the carbon-degradation pathway, genes involved in degradation of terpenes (chd but not Imo), glyoxylate (AceB), starch (amyA), chitin, hemicellulose, and fungal proteins were all significantly greater in low nitrate soils (Fig. 4). Finally, in the N reduction pathway genes involved in reduction of nitrate (nasA and narG), nitrite (nirK), and particularly nitric oxide (cnorB; cytochrome C nitric oxide reductase subunit B) were all significantly elevated in low nitrate N soils. Other genes presented in Fig. 4 (alpha galactosidase fungi, GAPDH Calvin, lmo-terpene, pcc, and sucD) were significantly associated with the set of high nitrate-N soils.

When grouped to family level, the abundances of 40 microbial taxa,

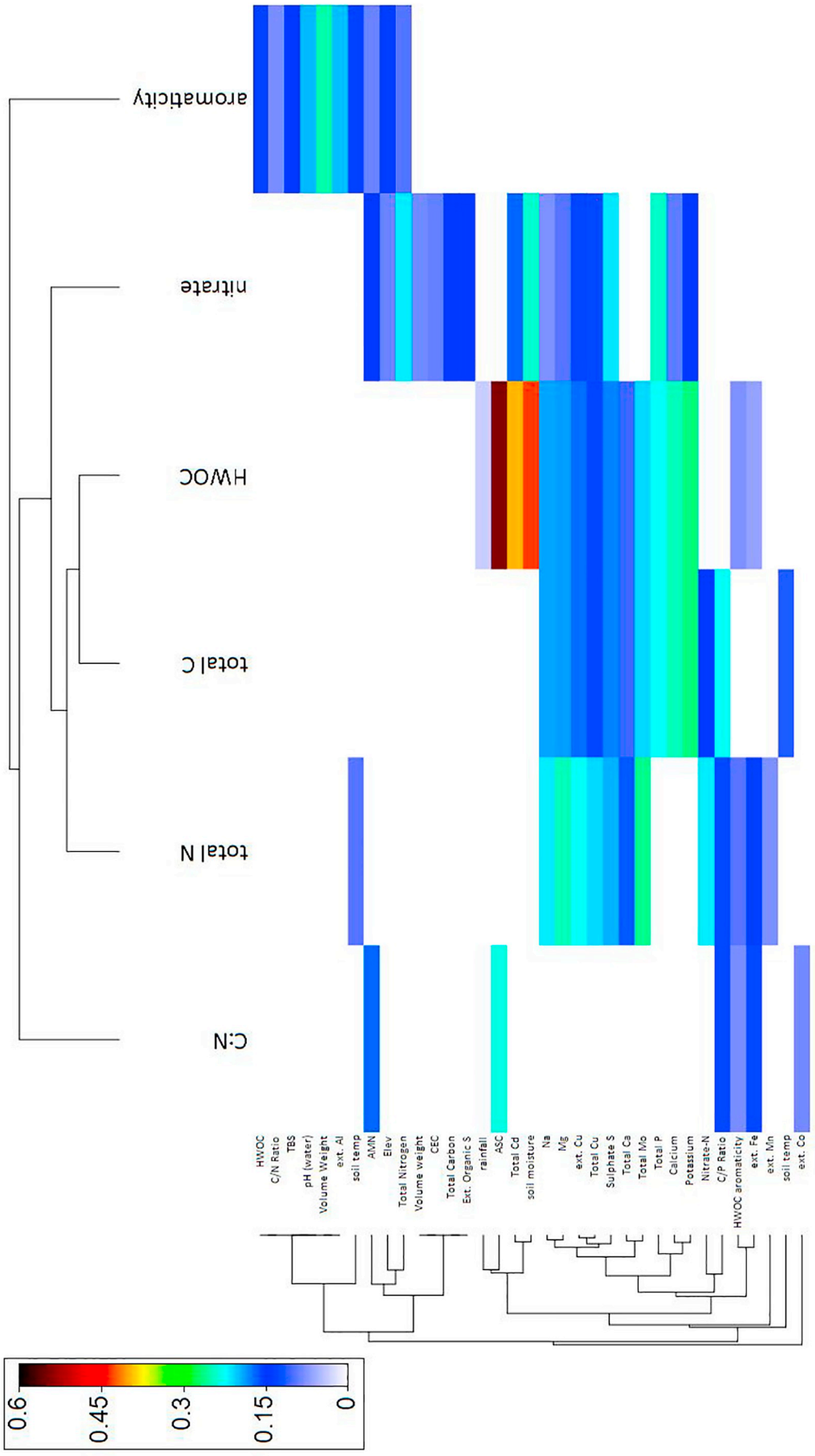

Fig. 2. Heat map showing degree of association between soil and environmental variables associated ($p < 0.05$) the C and N content of pasture soils. Values are R-squared (fit) values following linear regression.

Table 2
Soil and environmental variables that significantly differences between sets of soil that were identified as anomalously high or low in nitrate-N (relative to total N content).

Variable	ANOVA *p*-value†	Low nitrate-N soils		High nitrate-N soils	
		Average	Std. Error	Average	Std. Error
ASC[a]	0.040	57.07	4.93	44.077	3.56
Sulphate-S	0.033	5.71	1.24	10.77	1.76
AMN[b]/total N ratio	0.015	2.80	0.25	3.65	0.24
Nitrate-N	< 0.0001	25.45	4.16	94.80	9.12
C/N ratio	0.009	10.50	0.28	9.48	0.21
C/P ratio	0.014	64.00	8.86	37.77	3.87
E.C.[c]	< 0.0001	0.0414	0.0051	0.0846	0.0093
Extractable Fe	0.003	644.86	89.82	1121.92	141.42
Rainfall	0.010	3.20	0.33	5.34	0.789

† *p*-Value derived from permutation-based testing.
[a] ASC = anion storage capacity. EC = electrical conductivity.
[b] AMN = anaerobically mineralizable nitrogen.
[c] E.C. = electrical conductivity.

Table 3
Significant (permutation ANOVA) relationships between abundances of high-level gene categories between the relatively low- and high nitrate-N soil groups.

Variable	ANOVA *p*-value†	Low nitrate N soils		High nitrate N soils	
		Average	Std. Error	Average	Std. Error
Carbon degradation	< 0.001	−0.158	0.002	−0.173	0.004
Carbon fixation	0.028	−0.171	0.003	−0.180	0.004
Denitrification	0.006	−0.215	0.004	−0.238	0.007
Chitin synthesis	0.041	−0.169	0.054	0.024	0.082

† *p*-Value derived from permutation-based ANOVA testing.

covering several kingdoms/domains, were associated with relatively low or high nitrate N soils (Fig. 5). Typically, low nitrate-N soils had higher abundances of Actinobacteria, Firmicutes, and most orders of Proteobacteria, and several orders of fungi, and taxa within the Heterokonta and Cryptophyta. Across these, the largest fold-change differences in taxa between the low and high nitrate-N soils were in the abundances of Firmicute bacteria, such as the Clostridiales, Halanerobiales, and Erysipelotrichales, and the Proteobacterial-order Desulfobacterales. Note that the dual entries for Desulfobacterales relate to the families Desulfuromonadaceae and Desulfobacteraceae. The largest fold-change, and in which a positive value was reordered, was for the Desulfuromonadaceae. Large fold-changes in taxa abundance were also determined for Bangiales (phylum = Rhodophyta).

4. Discussion

Excess nitrate-N in agricultural soils is often undesirable. When concentrations exceed the ability of plants to assimilate N from soil, and when biogeochemical conditions are favourable, nitrate-N can move into waterways (Schlesinger, 2009), and/or be reduced via denitrification into potent greenhouse gases (Davidson, 2009; Erisman et al., 2011). In addition to potential environmental risk, N loss from soils represents an economical loss to farmers, reducing the profitability of agricultural system. As such, opportunities to retain nitrate in soils are being sought (Robertson and Vitousek, 2009).

To date, most studies investigating the microbiological processes underpinning soil nitrogen cycling, including formation and transformation of nitrate-N, have focussed on individual groups of N-cycling taxa. For example, the investigation of nitrate-N formation via oxidation of ammonia, or the sequential reduction of N through denitrification (e.g. Wakelin et al., 2013c). These studies have often been supported through application of molecular microbiology tools to understand the occurrence, abundance, and expression of metabolic genes associated with one or more steps in the N-cycling pathway; e.g. targeting the *amo*A gene for ammonia monooxygenase activity (Wakelin et al., 2013c; Tao et al., 2017). However, given the close coupling between the cycling of N and C, the rate or type of C-associated biogeochemistry in soils can affect N pools and flux (Soussana and Lemaire, 2014). These have impact not only for soil N availability, but for C sequestration (Shi et al., 2016). In order to assess the potential associations of both C and N cycling pathways on soil nitrate-N, we applied use of GeoChip microarray analysis. As such, this work is significant in both focus across both nutrient cycles, and also novel in the application of an ecological genomics approach across 50 well-characterised grassland (pasture) soils (Wakelin et al., 2013b).

In soils with levels of nitrate N high relative to the total N status, increased abundance of genes associated with degradation of fungal α-galactosidase were determined, along with reduced abundance of denitrification genes. Relative to the wider panel of 50 pasture soils, there were few differences in microbial taxa present (however see Fig. 5 for fine details). In contrast, soils with relatively low nitrate-N were characterised by wide ratios of C with other key nutrients (N, P, or S), lower sulphate-S, generally increased genetic potential for a range of C-cycling (degradation and autotrophy) and higher abundance of denitrification-associated genes. Changes in the functional potential of the

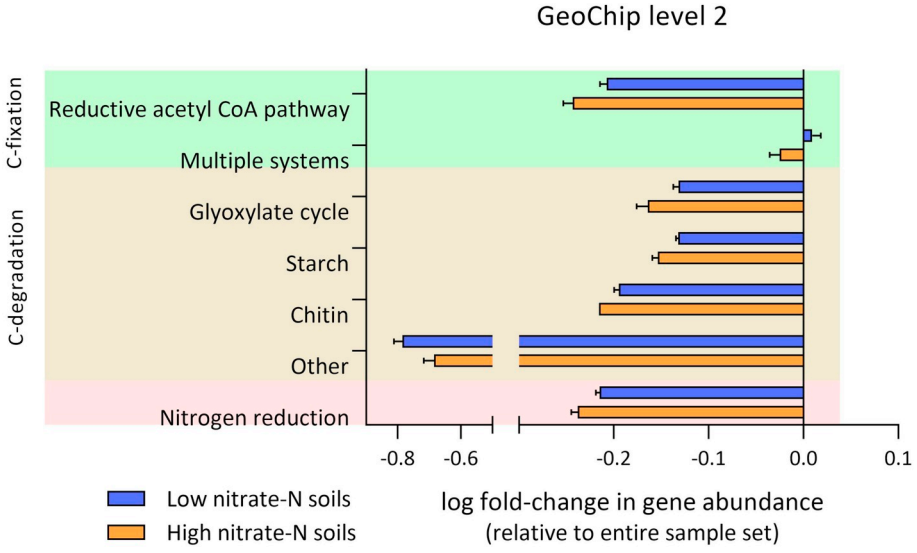

Fig. 3. Significant (permutation $p < 0.05$) variation in mid-level gene categories between groups of soil with relatively low or high nitrate N levels. Bars give means and standard errors of the means. For each gene, less negative values are in relatively higher abundance. 'Other' refers to decomposition of α-galactosidase by fungi. 'Multiple systems' refers to propionyl-CoA/acetyl-CoA carboxylase.

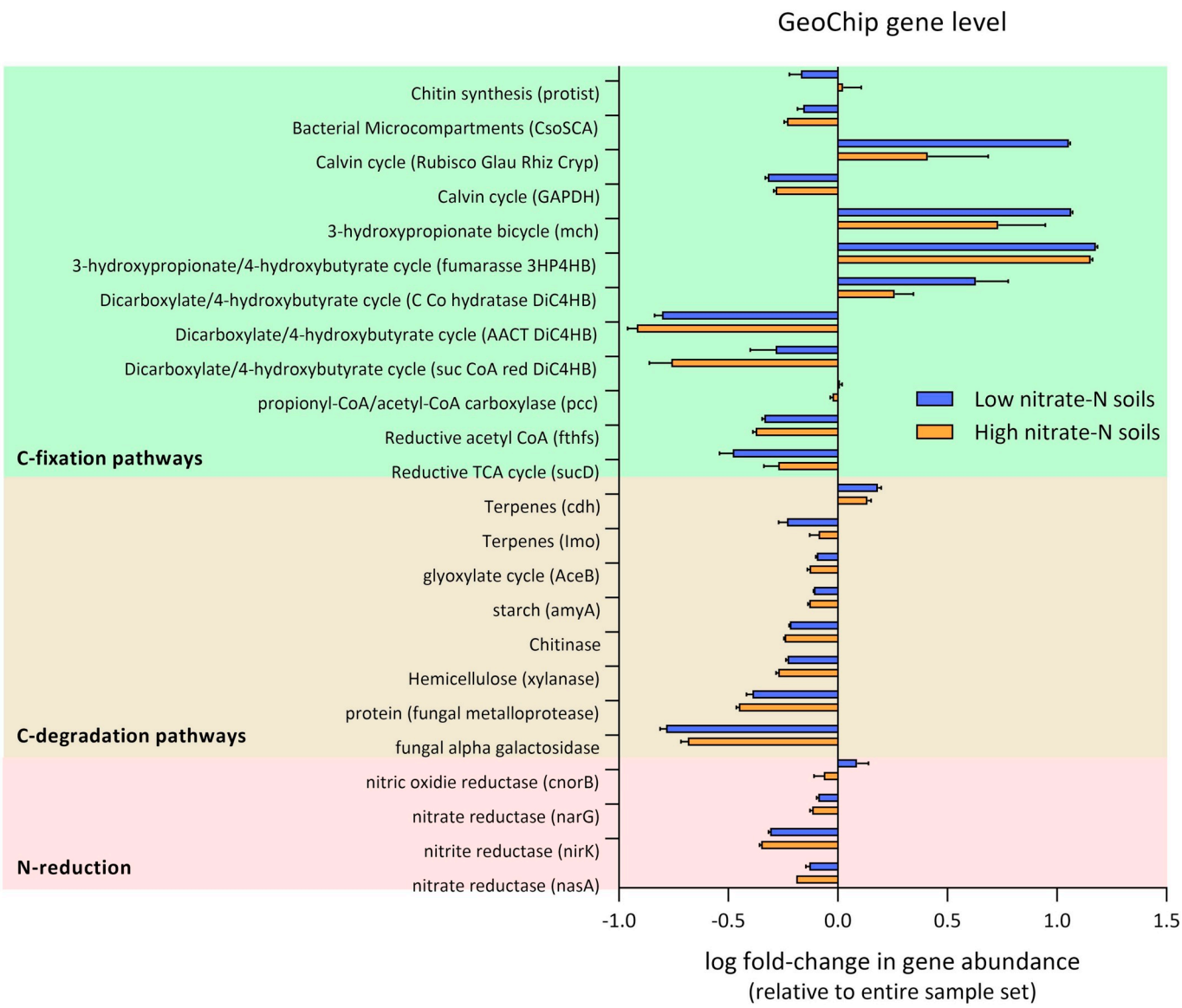

Fig. 4. Significant (permutation $p < 0.05$) variation in genes between groups of soil with relatively low or high nitrate N levels. Bars give means and standard errors of the means. For each gene, less negative values are relatively higher.

soil ecosystem (GeoChip data) were linked to increased Firmicute, Actinobacteria, and some Proteobacteria and fungal taxa. Elemental stoichiometry between carbon and other nutrients (N, P, and S) was strongly associated with decreased soil nitrate-N, demonstrating both the effect of nutrient ratios in affecting dynamics (e.g. Kirkby et al., 2011), and also fundamental association of the C-cycle as a driver of wider soil biogeochemistry.

The total C content, extractable organic C, C-aromaticity, and AMN (indicator of microbial biomass size; Wakelin et al., 2013a) of the soils were similar between the two treatment groups (*t*-tests $p > 0.05$). As such, effects related to influence of total soil carbon or the size of the microbial population on the nitrate-N contents in the soil could not supported. Rather, the group of soils with anomalously low-nitrate N had shifts in the genetic potential for different C-cycling pathways, particularly autotrophic C-fixation and C-degradation pathways (Fig. 4).

We inferred a greater potential for autotrophic C fixation in the low nitrate-N soils due to increased dicarboxylate-hydroxybutyrate and Calvin-cycling genes (Huber et al., 2008; Berg, 2011). Elevated potential for C-assimilation via CO_2 fixation may indicate C-associated stress within ecosystem, particularly given the wide ratios of C with other nutrients. These stoichiometric constraints to heterotrophic C cycling are well recognised (e.g. Kirkby et al., 2011) and potentially underpin this finding. Furthermore, while the relative contribution of microbial v plant-based autotrophic CO_2 fixation in to grassland soil ecosystems maybe low (e.g. Miltner et al., 2005), overall levels of microbially-derived C-inputs remain significant (Miltner et al., 2005; Ge et al., 2016), and are likely to have a disproportionally-important role in wider C-cycling (Yuan et al., 2012).

Changes in carbon degradation pathways among the anomalously high- and low-nitrate N soils were complex (Fig. 4). Overall, however, the results indicate a generally increased capacity for carbon degradation in soils having the anomalously low-nitrate N, included degradation of some terpenes (chd), glyoxylate (AceB; malate synthase), starch (amyA), chitin, hemicellulose, and fungal proteins. Of these, the association of some of these degradation pathways with fungal-associated carbon (chitin, fungal proteins) is noteworthy. With increasing C:N ratio, the proportion of fungal:bacteria in soil generally increases (Bardgett et al., 1996). This is supported in our study, as the C:N ratio of soils was positively associated with increased chitinase activity (i.e. fungal C-cycling). As these two groups of taxa have distinct physiologies, fundamental shifts in soil carbon cycling process are expected

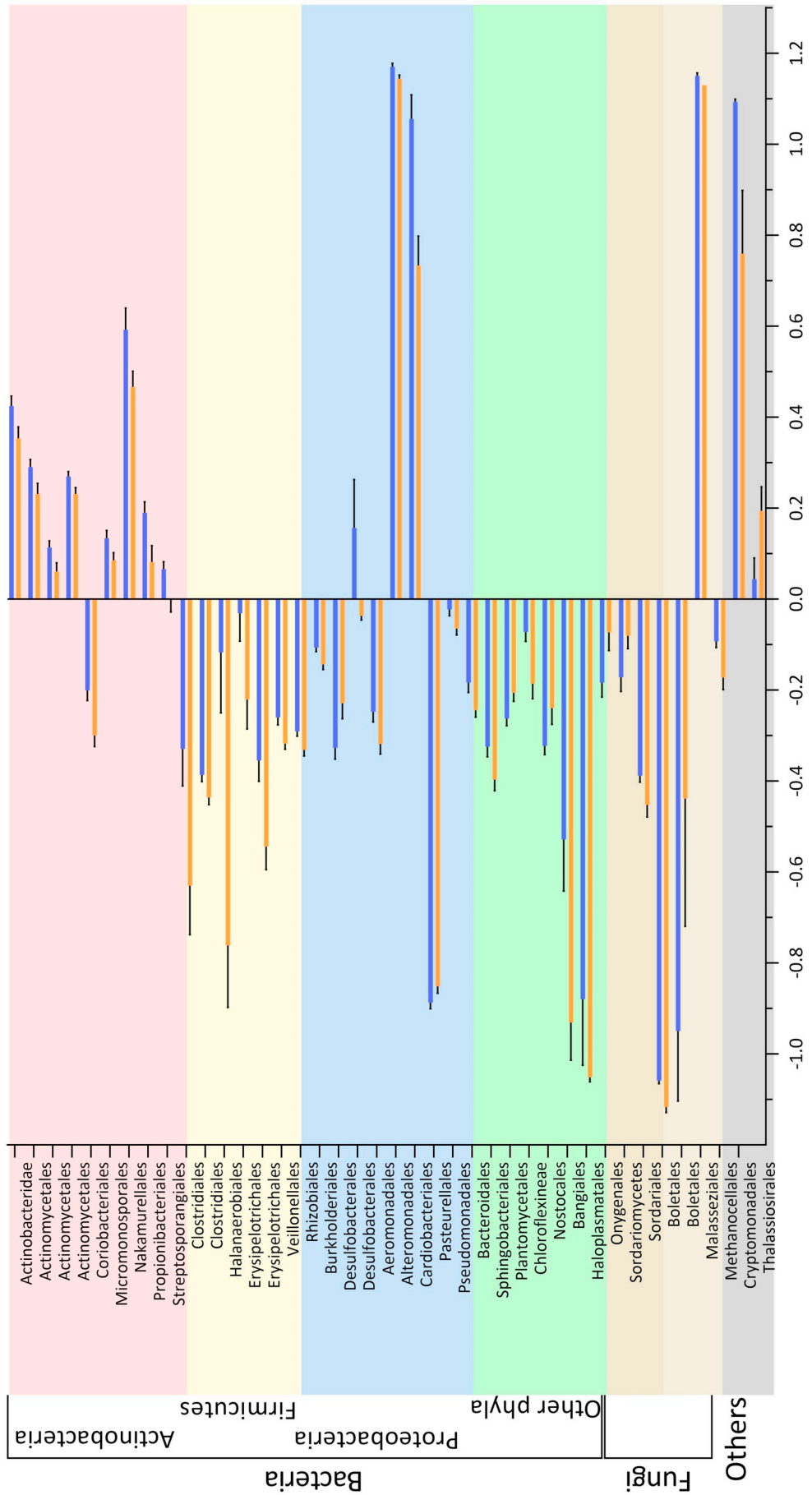

Fig. 5. Microbial taxa, extracted from GeoChip nucleotide accession data, significantly associated with relatively low or high nitrate-N soils. Others includes the Heterokonta (Thalassiosirales; diatom), Archaea (Methanocellales), and Cryptophyta (alt. Chromista; Cryptomonadales). Although data were formally tested at family level, the lowers taxonomic grouping shown here is 'order'. As such, there can be 2 entries for some Orders, as these relate to two different families of taxa. The full taxa dataset is available in the Supplementary information (Table S3).

(Waring et al., 2013; Malik et al., 2016), and these will extend to effects on the wider ecosystem cycling of both C and N (Schimel, 2013).

Our findings are of significance, as they suggest that alteration of inputs of soil C, or changes in soil C-cycling ecophysiology, may shift allocation of N between the total and mineral pools. Affecting C inputs, for example, maybe directed via by either direct or indirect approaches. Direct approaches are those associated with inputs of exogenous carbon to farmland soil, either through management of plant or animal residues (e.g. Tao et al., 2015), or via supplementation of soil with simple or complex carbon sources (e.g. molasses). However, unless these can be maintained indefinitely, effects may be short-lived and the approach unsustainable or uneconomic in broad-acre grassland systems. In comparison, indirect (or secondary) management maybe achieved through quantitate or qualitative shifts in carbon exuded into the rhizosphere (rhizodeposition), particularly as this represents the largest input of C into the ecosystem (Jones and Donelly, 2004), and directly incorporates C into the soil matrix (Balesdent and Balabane, 1996). This may be achieved through management of the botanical composition of the pasture sward, breeding pasture plants for specific below-ground traits (e.g. altered C-deposition), or utilising novel endophyte symbiosis to alter root exudation chemistry (e.g. Wakelin et al., 2015). Finally, shifts in mycorrhization of plant communities have been shown to shift soil ecosystem carbon dynamics (Clemmensen et al., 2015). This opens the tantalizing potential to manage soil communities, e.g. through delivery of specific mycorrhizal species to grasslands or breeding plants for specific fungal associations, to alter soil C cycling and N dynamics.

Previously in New Zealand grassland soils, we have found that microbial community structure is mostly closely associated to variation in the pH and sulphate-S status (Wakelin et al., 2013a). As the combination of both variables explained the largest proportion of microbial community variation, it suggests that the effects of pH and SO_4-S are largely independent of each other, and therefore that SO_4-S status of soil is an important driver of community assemblage in grassland soils. Change in soil pH status has been linked, through land use change, on abundances of Firmicutes, Actinobacteria and Bacteroidetes (Jesus et al., 2009), and Acidobacteria (Rousk et al., 2010; Lauber et al., 2008). However, as there was no significant difference in the pH between the soils low in nitrate-N and the wider group, the differences in these taxa were closely associated with variation in the C and N cycling functions. Indeed, this is axiomatic given the taxonomic data were extracted from the underlying GeoChip C and N datasets. This information provides further insights into the ecology of bacterial groups, at high taxonomic levels; i.e. our data indicate that these taxa have an important role in C and N cycling in systems where the nutrient cycles are closely coupled. These results provide deeper information on the potential ecological roles of these Phyla, beyond describing the habitat space they constrained to by pH range etc. For example, Firmicutes were not associated with changes in soil fertility status (Wessén et al., 2010), however this may be due to pH limiting the range of occurrence, and not a wider reflection of their underlying importance in various biogeochemical processes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2019.04.012.

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