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Bioactive carbon improves nitrogen fertiliser efficiency and ecological sustainability

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Agriculture's most pressing challenge is raising global food production while minimising environmental degradation. Nutrient deficiencies, principally nitrogen (N), limit production requiring future increases in fertiliser use and risk to proximal non-agricultural ecosystems. We investigated combining humate with urea, globally the most widely used N-supplying fertiliser, in a four-year field study. Humate increased pasture yield by 9.8% more than urea and significantly altered soil microbial diversity and function. Humate increased N retention suggesting microbial sequestration may lower N leaching and volatilisation losses. Humic microbial bio-stimulation could feasibly increase fertiliser efficiency and development of ecologically sustainable agriculture.

It is estimated one billion people currently suffer from chronic hunger and that global food production needs to double when the global population will stabilise at around 9.8 billion in 2050^{1,2}. Increases in agricultural output have been largely driven by raising the use-efficiency of labour, land, capital and other inputs, including fertilisers^{3,4}, but further escalation in food demand will place increasing pressure on finite land and water resources, raising serious concerns over land use intensification and global food security^{5–7}. The challenge of meeting sustainable food production while achieving the UN Millennium Development Goals of ending hunger and ecosystem protection is substantial^{8–10} and has been described as “a perfect storm”¹¹.

Raising global food production will unavoidably require increases in fertiliser inputs, principally nitrogen (N) and phosphorus (P)^{12,13}. N is essential in all biological processes as a primary component of nucleotides and proteins and is a principle factor determining ecosystem productivity. N supply is frequently insufficient in terrestrial systems for full realisation of potential plant growth, hence its fundamental importance in agricultural and horticultural production. Throughout history N fertiliser application has been used to increase the productive efficiency of land and it is a major component of the “Green Revolution” responsible for meeting global food demand for about half of the world's population^{12,14,15}.

Inert nitrogen comprises 78% of the atmosphere, comprising a substantial ecological resource, but is only made biologically available by lightning or, more commonly, by N-fixing micro-organisms. Development of industrial synthesis of ammonia using atmospheric N occurred early in the 20th century leading to production of abundant and cheap fertilisers, principally urea, and subsequent widespread adoption of their use. Intensive use of agricultural synthetic N has significant environmental costs^{16,17}. Nitrogen is highly mobile in soil solution and readily moves from agro-ecosystems via unmanaged pathways, influencing non-agricultural ecosystems¹⁴. Atmospheric N from agricultural and other land uses affects the global climate, comprising 81% of human derived nitrous oxide (N₂O) emissions during 2007–2016 and contributing around 28% of the total net anthropogenic greenhouse gas emissions¹⁸. Localised atmospheric N deposition also affects natural biodiversity and ecosystem function¹⁴. Leached inorganic N creates major aquatic environmental problems by a variety of pathways, acidification of unbuffered freshwater ecosystems and eutrophic stimulation of primary producers in freshwater and marine systems, which can result in anoxia or toxicity and significant biological impacts. The required increase in global agricultural fertiliser is forecast to cause 2.4- to 2.7-fold increases in nitrogen- and phosphorus-driven eutrophication of terrestrial, freshwater, and near-shore marine ecosystems, with significant loss of ecosystem services¹⁹. While there is no simple solution, scientific and technological innovation remain a key strategy for implementing sustainable intensification, defined as producing more food from the same land area while reducing environmental impacts²⁰.

It has long been known that soil microbial communities have a key role in mediating ecosystem processes, notably soil structure and nutrient turnover²¹, but developments in quantitative genomic analysis have facilitated

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approaches for tackling previously difficult investigations of plant-microbiome dynamics^{22,23}. Recent studies have shown how closely microbial diversity and function are coupled with plant productivity via root exudates, nutrient cycling and uptake, plant growth processes and climate change^{24–26}.

Humic substances are naturally occurring organic compounds arising from the decomposition of plant, animal and microbial residues and many studies have reported beneficial effects on plant processes and productivity^{27–30}. Other studies have reported neutral or negative responses, uncertainty regarding modes of action and inconsistency in effectiveness^{31–34}. Reviews repeatedly note the scarcity of evidence from robust long-term field trials, resulting in uncertainty regarding recommendation for commercial application^{35–37}.

Advances in analytical technology have also opened new approaches to investigate humic substance chemistry providing tools for explicit molecular evaluation of their bioactivity^{38–40}. This has resulted in fundamental reinterpretation of previous analytical misinterpretations of humic substances^{39,41} and holds significant potential for clarifying understanding of response pathways, a major requirement for determining future practical applications.

Another technological innovation, particulate coating of N fertilisers, has recently been advocated as a potential solution for improving fertiliser efficiency, with studies reporting that coated urea improved N release and reduced N loss^{42–44}.

We hypothesised that coating urea with humic carbon may act as a microbial bio-stimulant, improving plant nutrient acquisition. To test this we designed a field experiment in developed pasture grassland applying a standard rate of urea fertiliser, urea coated with humate and urea and P-supplying guano coated with humate. We measured monthly or bi-monthly herbage production over four growing seasons from 2014–2018 and assessed microbial diversity and activity microscopically and by DNA amplification for fungi, bacteria and gammaproteobacteria, bacteria with genes for nitrogen oxidising capability.

Methods

Study site. The study was located at Agland Farm, Mataura, New Zealand (46° 48'33S; 168° 48'04E) at 141 m above sea level in fertilised pasture, principally consisting of ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). The climate is temperate with a mean annual temperature of 10.6 °C, ranging from 14.8 °C in summer to 4.6 °C in winter, and a mean annual precipitation of 916 mm (2015–2018). The soil is a Waimumu silt loam, rolling phase classified as an imperfectly to poorly drained Mottled Fragic Pallic/ Perched Gley Soil⁴⁵ with moderate to slow permeability. The upper topsoil (0–15 cm depth) had a pH of 6.1, a total carbon content of 4.5%, a total nitrogen content of 0.30%, Olsen extractable phosphate of 7 mg/L, and a base saturation of 63.0%. The site has been used for over 50 years for pasture grazing and cultivated for fodder crop production. It was last fertilised 18 months before trial commencement with 125 kg/ha superphosphate and 1 tonne/ha lime.

Materials. We used commercially available urea from Ballance Agri-Nutrients and Southern Humate, mined from the Kapuka K1b lignite seam at Waituna, Southland. Humate was fine ground to <2 mm and mixed with granular urea and lime immediately prior to application. Total carbon content averaged 56.45 ± 0.05% (standard error of the mean), humic acid 36.7 ± 2.0%, fulvic acid 2.03 ± 0.08% and cation exchange capacity (CEC) was 125.5 ± 0.0 me/100 g.

Experimental design and sampling. We initially investigated pasture responses to five rates of humate with urea and guano in the first growing season (2014–2015) using a randomised complete design block design with eight fertiliser applications in five blocks, totalling 40 plots with 5 replications per treatment. Treatments were nil fertiliser, five rates of humate and urea applied monthly, and one rate of humate and urea applied bimonthly with or without guano. Humate rates were 0, 2.5%, 5%, 10% and 20% of the weight of urea, applied initially as 60 kg/ha of urea with 6 kg/ha of lime on the 28th October 2014 and then applied monthly as 50 kg/ha of urea with 5 kg/ha lime from November 2014 to February 2015. The bi-monthly 10% humate rate was applied ± 100 kg/ha guano. Total seasonal urea applied varied from 160 to 260 kg/ha per treatment.

In the following growing seasons we examined responses using urea with the two highest yielding rates of humate with or without lime. The experimental design was a randomised complete block with five fertilisers in five blocks with a split-plot lime treatment ± 5 kg/ha, totalling 40 plots with five (nil) or eight or nine main treatment replicates with four or five sub-treatment lime replicates. Treatments were nil fertiliser without lime, urea at 50 kg/ha ± lime, urea at 50 kg/ha with 5 kg/ha humate (10%) ± lime, urea at 50 kg/ha with 10 kg/ha humate (20%) ± lime, and urea at 50 kg/ha plus 5 kg/ha humate and 50 kg/ha guano ± lime. Fertilisers were applied ~ bimonthly four times each season between November and April, totalling 200 kg/ha urea in the second and third seasons but were only applied once, in spring of the last season (October 2017, totalling 50 kg/ha urea), to examine the longevity of fertiliser effects. Plot size was 10 m² separated by 1 or 2 m buffer zones.

Pasture production assessment. Pasture yield was determined by harvesting with rotary mower to a 4–6 cm cut height, except for 2–3 cm in April 2017, and weighed to an accuracy of 5 g. Herbage was evenly returned and fresh weight sub-samples were taken from every plot, weighed to an accuracy of 0.02 g and dried to constant weight for calculating dry matter (DM). There were five approximately monthly assessments during the initial growing season (November 2014 - March 2015), eight in the second (September 2015 - April 2016), six in the third (September 2016 - April 2017) and three ~bimonthly assessments in the final season, when determining the longevity of fertiliser effects (October 2017 - March 2018).

Microscopic assessment of soil microbiology. Fertiliser effect on seasonal changes in fungal and bacterial abundance and activity were determined in spring immediately before fertiliser application (27th October 2014) from a bulk sample of forty 2.5 × 7.5 cm topsoil cores, in summer (3rd February 2015) from a bulk sample of four cores per plot and in autumn (20th March 2015) from ten cores per plot, totalling 40 samples per

assessment. Microbial biomass and activity was determined using epifluorescent and phase contrast-differential interference contrast microscopy by Soil Food Web New Zealand⁴⁶.

DNA assessment of soil microbiology. To assess fertiliser effect on microbiology after two seasons, three topsoil cores were extracted as previously from every plot on the 9th January 2017, providing 40 bulked samples for DNA analysis, and frozen at -20°C until laboratory processing. Soil was thoroughly mixed and a 12 gram sub sample was shaken with 50 ml of sterile water then placed into an ultrasonic bath for 3 minutes. A 2 ml extract was centrifuged for 15 minutes at $11,400 \times g$, the supernatant removed and the residue stored at -20°C . DNA was extracted using DNeasy PowerSoil kit (Qiagen protocols), concentration measured by spectrophotometer using a Nanodrop[™], and stored at -20°C . This was replicated three times giving a total of 120 assays. Possible detection errors were checked, and if necessary corrected, by examination of replicate consistency.

Microbial DNA in soil samples was determined using Denaturing Gradient Gel Electrophoresis (DGGE) analysis by amplification using three PCR primer sets: the total bacterial communities identified using the V3 hyper-variable region of the 16S rRNA gene with primers 341 F GC and 518R⁴⁷; the total fungal communities using the primer pair AU2 and AU4 and the PCR product from the primer pair AU2-AU4 used as template for a second nested PCR with primer pair FF390 and FR1-GC^{47,48}; and the gammaproteobacteria communities using a nested PCR with primers (Gamma395F - Gamma871R and 518F GC - 785R)⁴⁹. Diversity of ammonium oxidising bacteria was similarly determined using amplification of the ammonia monooxygenase subunit A gene (*amoA*) with primers amoA1F-GC and amoA2IR⁵⁰. Phoretix 1D Pro software measured DNA band profiles.

Statistical analysis. The statistics package R 3.6.2 was used for data analysis⁵¹. The Shapiro-Wilk and Kolmogorov-Smirnov tests examined assumptions of normality for modelled distributions. If required, the data were further analysed using negative binomial general linear models. Analysis of variance and regression using linear or generalised linear models was used to test fertiliser effects with Tukey tests for pairwise comparisons. Analyses were also run with Welch F or Kruskal-Wallis tests not assuming equal variances or normal distributions. Chi square and Fisher's exact test of independence were used to test the probability of observed responses. Fertiliser effect on microbial population composition was assessed by multivariate canonical correspondence analysis and multivariate general linear models with resampling using the vegan and mvabund packages^{52,53}.

Results

Fertiliser application significantly increased pasture dry matter (DM) production, averaged across four growing seasons ($F = 6.06$, $P < 0.00001$), with a significant difference between seasons ($F = 26.39$, $P < 0.00001$) with no fertiliser \times season interaction ($F = 0.32$, $P < 0.98$; Fig. 1a). In the first growing season (2014–15) urea increased average production by 47% compared with nil fertiliser application in unfertilised grassland, urea with 10% humate by 70%, urea with 20% humate by 59% and urea with 10% humate and gauno by 42% ($F = 13.4$, $P < 0.001$, Fig. 1b). By the final growing season (2017–18) urea increased production by 8%, urea with 10% humate by 17%, urea with 20% humate by 13% and urea with 10% humate and gauno by 12% ($F = 1.08$, $P < 0.37$, Fig. 1d). In the twenty two assessments between 2014 to 2018 addition of 10% humate to urea gave greater production than urea in every assessment (Chi square = 44.0, $P < 0.0001$; Fisher's Exact Test $P < 0.0001$) and 20% humate gave greater production in 20 assessments (Chi square = 36.7, $P < 0.0001$; Fisher's Exact Test $P < 0.0001$). Lime addition did not significantly change production in any season, or with any fertiliser (54.9 vs 55.3 kg DM/ha/day; $F = 42.4$, $P < 0.922$).

Increasing humate rate significantly linearly increased production by March and September in the first year of the trial ($\text{DM} = 0.256 * \text{Humate \%} + 38.5$, $F = 4.49$, $P < 0.045$; $\text{DM} = 0.126 * \text{Humate \%} + 7.4$, $F = 4.50$, $P < 0.045$) with variability within treatments masking differentiation between linear and curvilinear responses.

Fertiliser applied by summer of 2015 continued to affect pasture production after seven and a half months, though non-significantly ($F = 2.45$, $P < 0.079$), before production sharply fell towards unfertilised pasture a month later (Fig. 2a). In both the initial and final growing seasons (Fig. 2a,b) humate addition produced higher yields than from urea, notably in the spring of the 2015–16 growing season where the production difference widened from 6% and 16% (at the 10% and 20% humate rates) in the autumn of the 2014–15 season to 31% and 41% in early spring of the 2015–16 season. In the final growing season these humate rates lifted production above urea by 12% and 20% (Fig. 2b).

Total bacterial and fungal biomass and activity varied during the first growing season (2014–15) decreasing from spring to summer then increasing in autumn ($F = 5.35$, $P < 0.001$, bacteria $F = 5.25$, $P < 0.007$, Fig. 3a,b; active bacteria $F = 28.4$, $P < 0.0001$; active fungi $F = 17.84$, $P < 0.0001$; Fig. 4c,d). Rate of humate application significantly affected only fungal activity ($F = 2.99$, $P < 0.03$).

By the third growing season, in January 2017, fertiliser application differentially affected microbial populations (Fig. 4). Fertiliser significantly altered both the number of co-occurring taxa (chi-squared bacteria = 113.4, $P < 0.00001$; fungi = 60.4, $P < 0.0067$; = 76.7, $P < 0.0001$; Fig. 5a) and the total number of taxa ($F = 2.95$, $P < 0.022$; Fig. 5b). Fertiliser was significantly related to two specific bacteria operational taxonomic units, five fungal taxa and one gammaproteobacteria taxa at the $P < 0.05$ level of significance, one bacterial taxa at the $P < 0.01$ significance level and one fungal taxa at the $P < 0.001$ significance level in generalised linear models. Fertiliser also significantly affected the distribution of bacteria with ammonia oxidising genes in which occurred in all urea treatments, once in a urea plus humate plus guano treatment but were absent on all nil fertiliser, 10% and 20% urea plus humate treatments (chi-squared = 15.143, $P < 0.004$, Fisher's Exact test $P < 0.001$). Pasture production in January was significantly related to composition of fungal taxa (multivariate general linear model, Likelihood Ratio Test LTR = 103.8, $P < 0.02$) and gammaproteobacteria (LRT = 60.1, $P < 0.007$) but not to bacteria populations (LRT = 24.8, $P < 0.29$).

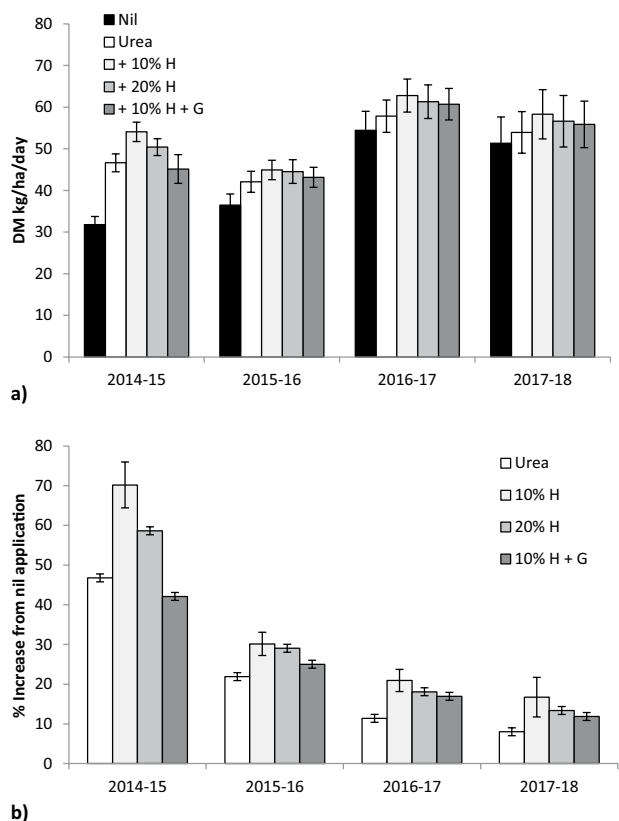


Figure 1. (a) Effect of urea, humate (H) and guano (G) addition to urea on pasture dry matter (DM) production 2014–15, 2015–16, 2016–17, 2017–18 growing seasons; (b) Change in production relative to unfertilised grassland, mean \pm standard error.

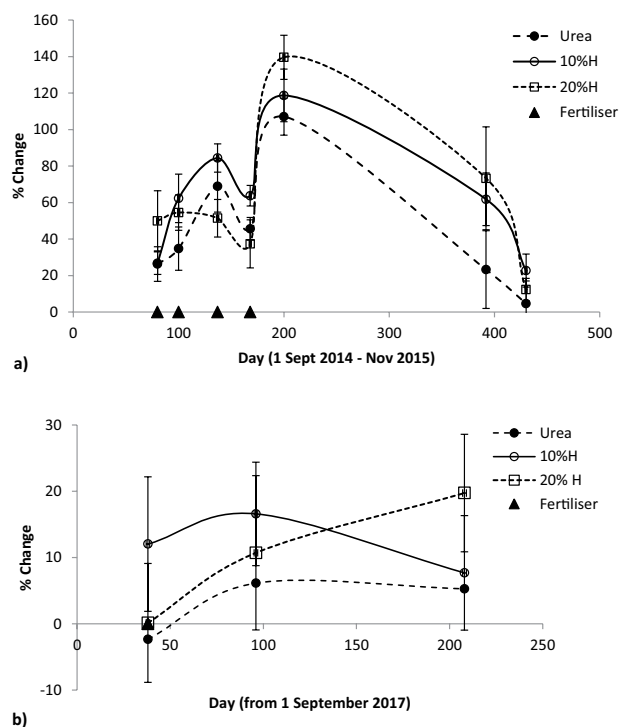


Figure 2. Effect of humate application rate on pasture production in March and September 2015 and 2014–2018, mean \pm standard error. Longevity of urea and humate (H) effect on pasture production relative to unfertilised pasture (a) after summer application of the 2014–15 season to the start of the 2015–16 growing seasons and (b) after spring application in the 2017–18 season, mean \pm standard error.

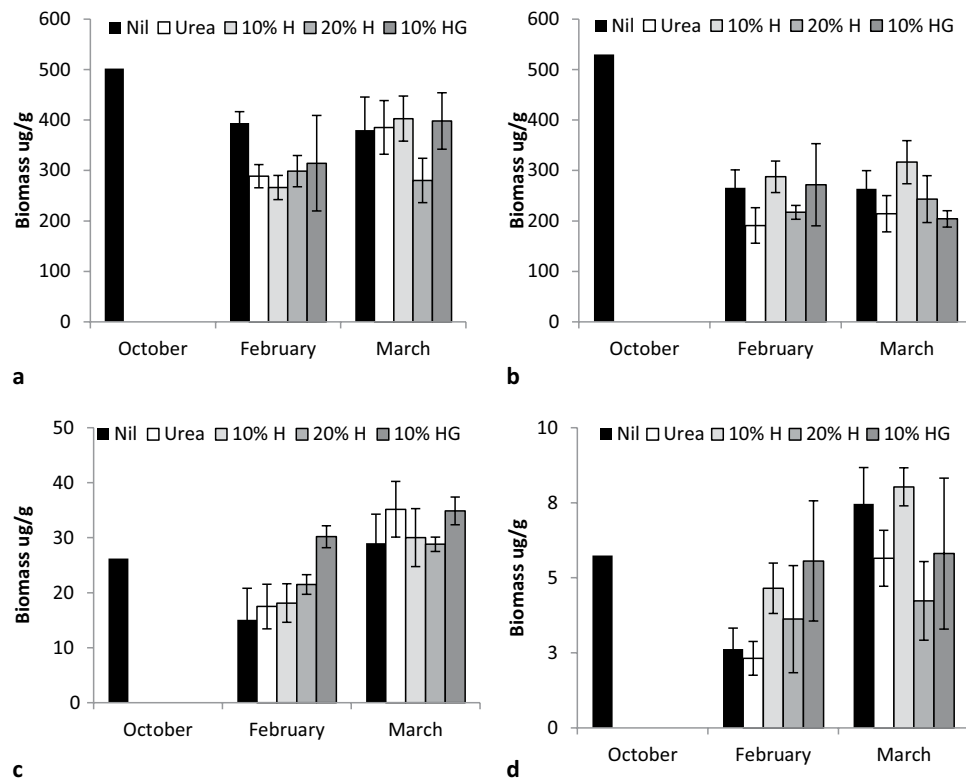


Figure 3. Effect of urea, humate (H) and guano (G) addition to urea on (a) total bacterial biomass and; (b) total fungal biomass (c) active bacterial biomass and (d) active fungal biomass, October 2014 to March 2015, mean \pm standard error.

Discussion

N deficiency limited pasture production, as shown by the 22.9% average increase with urea across all seasons, which is consistent with previous Southland studies^{54,55} and responses on many global soils^{14,16}. Humate addition increased production by a further 9.8%, a magnitude which is agriculturally significant and demonstrates superior N use efficiency of production per unit of applied N. The increase in average yield with humate was similar to the 9–14% increases reported in other New Zealand⁵⁶ ryegrass - white clover humate trials. Ten percent humate addition gave the greatest rate response while 20% gave a slightly lower non-significant response, possibly indicating a small inhibitory rate effect or this may simply be due to experimental variability.

The effect of humate was consistent and persistent, most notably over winter in 2015 where 10% and 20% humate increased early spring production by 31% and 41% more than urea, before soil N pools were depleted and yield declined in late spring. This response, occurring when plant demand for N is increasing but soil available N is limiting, showed that humate addition resulted in enhanced supply of N and possibly other nutrients. If this was due to greater retention of summer applied N over late autumn - winter when the risk of nitrate leaching and nitrous oxide emissions are greatest due to high rainfall and soil moisture levels, low temperatures, slow plant growth and low nutrient demand^{57,58}, humate may confer environmental benefit by reduce the quantity of N available for leaching or volatilisation, possibly by sequestration in microbial biomass^{59,60}.

The yield increase with humate is consistent with beneficial effect of humic substance reported in other pasture species trials. Ryegrass shoot yield increased by 150% on oxisols with humic addition while red clover (*Trifolium pratense* L.) showed only a small increase, attributed to differences in P mobilisation between the species⁶¹. Growth of Italian ryegrass (*Lolium multiflorum* Lam.) and lucerne (*Medicago sativa* L.) was significantly increased with Australian lignite-derived humates⁶². Humic substances have significantly increased growth many other non-pasture species in many other studies^{30,35,36}. The reason for the progressive decline in relative response to fertiliser application during this trial is not known, but cannot be attributed to N accumulation from urea as production increased in unfertilised pasture. It may be due to factors related to cessation of mechanical disturbance or enhanced biological N production.

Fertiliser significantly affected the biomass and composition of soil fungal populations in the first season though the variability associated with microscopic assessment may have masked detection of differences in other populations. Pasture yield in the third season was significantly correlated with the taxonomic diversity fungi and gammaproteobacteria populations, determined by more sensitive DNA amplification, and fertiliser application was significantly related to individual fungal, bacterial and gammaproteobacterial taxa. This showed that microbial interaction is a causal factor related to plant production and that humate affected some interactions differently from urea, particularly as the effects with cannot be attributed to the quantity of macro or micronutrients humate contained. Humate acted as a bio-stimulant affecting both microbial diversity and function of both fungal

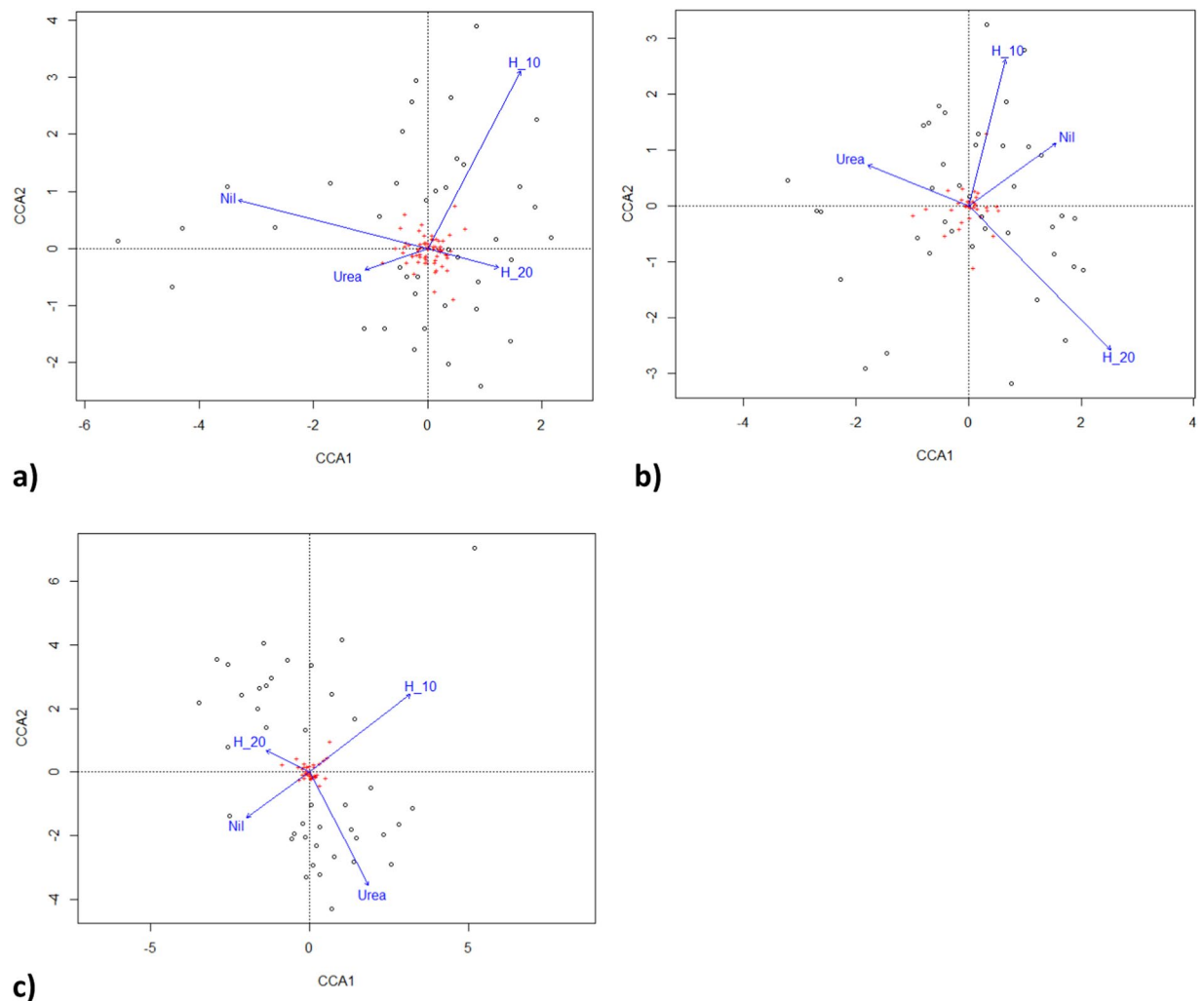


Figure 4. Canonical correspondence analysis of urea and humate rate (H) effect on (a) fungi, (b) bacteria, (c) gammaproteobacteria. Blue vector lines indicate direction and strength of fertiliser interactions, circles = fertiliser plots, crosses = taxa, H = % Humate.

and bacterial taxa. Humic substances have previously been shown to affect rhizosphere microbial populations^{63–65} and humate may act similarly to humic extracts known to increase legume nodulation and nitrogenase activity, stimulate *nod* gene expression, cell metabolism and nutrient transport⁶⁶. Another possible mode of action is through stimulation of microbial production of plant growth regulators such as auxins, cytokinins or abscisic acid if the appropriate humic precursors are present in populations^{67–69}. Humate may also affect nutrient or micronutrient availability or uptake as humic substances have been shown to increase the abundance of micronutrients, root hair development and nutrient absorptive capacity, as well as stimulating gene expression and ion uptake activity in root membranes^{70–73}. Humate may also indirectly affect plant growth processes by increasing aggregate stability and improving soil structure⁷⁴.

The lower yield responses with guano addition showed that the phosphorus it supplied did not increase production, indicating that N rather than P deficiency was the principal factor limiting pasture growth. The difference in response was greatest in the first growing season when guano was only applied bimonthly, and the rate of solubilisation of organic P release compared to rapid inorganic N release from urea may have affected production responses.

The general differentiation in microbial populations between urea and urea plus humate is supported by the significant difference in functional genes specific to ammonium oxidation. This shows that humate directly influences microbial metabolic N function and that plant responses cannot be solely due to carbon coating of urea granules reducing N volatilisation losses and hence improving utilisation^{42,43}. The effect of humate on gammaproteobacteria populations, ecologically important for having genes for the oxidation of nitrite and nitrate, critical processes in soil N cycling, suggests that humate may affect soil N supply and plant nutrition through this pathway.

Recommendation for the commercial applicability of humic substance has been equivocal due to inconsistency in plant field responses, uncertainty regarding modes of action, and scarcity of scientifically robust

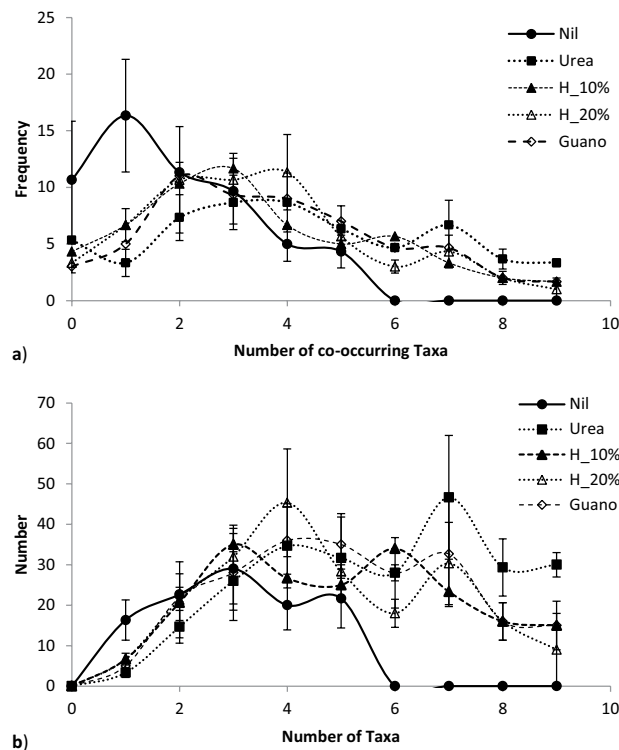


Figure 5. Effect of urea, urea plus humate (H) and urea plus humate and guano on (a) the number of co-occurring combined microbial taxa and (b) total number taxa per sample, mean \pm standard error.

long-term field trials^{35–37}. Our results support the agricultural use of humate and suggest that failure to adequately consider microbial interactions, inadequate experimental sensitivity and insufficient experimental duration may be contributory factors in negative evaluations of humic substances. Variability in individual pasture response in the first season caused us to increase main effect replication in the remaining seasons, but despite careful assessment natural field variability still remained, masking experimental sensitivity to detect subtle fertiliser effects. Our bulk sampling in the initial assessments of microbial biomass provided a general, but relatively coarse assessment of soil microbiology, rather than more focused specific assessments, e.g. from the rhizosphere of individual pasture species, which undoubtedly contributed to experimental variability, leading to the application of more precise DNA assessment. Variation in the composition and activity of humic substances has long been recognised as a major factor contributing to inconsistency in plant responses^{35,75,76} and these results with Southern Humate cannot be uncritically generalised to all humic products.

Improving the effectiveness of urea by coating with bioactive humic carbon opens new possibilities for improving global sustainable agriculture. The bio-stimulation of the soil microbiome we observed with a pasture production system would almost certainly extend to other production systems, given the beneficial responses to humic substances already recorded across a wide range of crop and horticultural species^{30,35,36,37}. The low cost, resource abundance and simplicity of incorporation with humate provides a feasible, easily implementable fertiliser technology with potential to improve N efficiency for substantial agronomic and environmental benefit.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 17 July 2019; Accepted: 3 February 2020;

Published online: 24 February 2020

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Acknowledgements

This study was funded by Callaghan Innovation and Southern Humates Ltd. We thank Chris Nel for technical support in measuring pasture production; Dr Neil Mitchell and Trevor Webb for reviewing the manuscript.

Author contributions

P.E. designed and supervised the trial, did the statistical data analysis and wrote the manuscript. H.R. performed the DGGE assays and data analysis and reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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