Ecological stoichiometry controls the transformation and retention of plant-derived organic matter to humus in response to nitrogen fertilisation

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Abstract

Carbon (C) sequestration in soils is a means for increasing soil organic carbon (SOC) stocks and is a potential tool for climate change mitigation. One recommended management practice

to increase SOC stocks is nitrogen (N) fertilisation, however examples of positive, negative or null SOC effects in response to N addition exist. We evaluated the relative importance of plant molecular structure, soil physical properties and soil ecological stoichiometry in explaining the retention of SOC with and without N addition. We tracked the transformation of ^{13}C pulse-labelled buffel grass (*Cenchrus ciliaris* L.), wheat (*Triticum aestivum* L.) and lucerne (*Medicago sativa* L.) material to the $< 53\ \mu\text{m}$ silt+clay soil organic C fraction, hereafter named “humus”, over 365-days of incubation in four contrasting agricultural soils, with and without urea-N addition. We hypothesised that: a) humus retention would be soil and litter dependent; b) humus retention would be litter independent once litter C:N ratios were standardised with urea-N addition; and c) humus retention would be improved by urea-N addition. Two and three-way factorial analysis of variance indicated that ^{13}C humus was consistently soil and litter dependent, even when litter C:N ratios were standardised, and that the effect of urea-N addition on ^{13}C humus was also soil and litter dependent. A boosted regression analysis of the effect of 44 plant and soil explanatory variables demonstrated that soil biological and chemical properties had the greatest relative influence on ^{13}C humus. Regression tree analyses demonstrated that the greatest gains in ^{13}C humus occurred in soils of relatively low total organic C, dissolved organic C and microbial biomass C (MBC), or with a combination of relatively high MBC and low C:N ratio. The greatest losses in ^{13}C humus occurred in soils with a combination of relatively high MBC and low total N or increasing C:N ratio. We conclude that soil variables involved in soil ecological stoichiometry exert a greater relative influence on incorporating organic matter as humus compared to plant molecular structure and soil physical properties. Furthermore, we conclude that the effect of N fertilisation on humus retention is dependent upon soil ecological stoichiometry.

47

48 **Keywords**

49 Soil organic carbon, nitrogen, decomposition, ¹³C-labelled organic matter

50

51 **1. Introduction**

52 Soils under agricultural management have 25 – 50% lower soil organic carbon (SOC) stocks
53 compared to soils under native vegetation (Lal, 2008). This is a combined result of the
54 removal of native vegetation, physical disruption of soil macro-aggregates, increased
55 oxidation of humified SOC, soil erosion and decreased OC inputs (Paustian et al., 1997).
56 Sequestration of OC may be an important means toward restoring depleted SOC stocks, and
57 for mitigating rising atmospheric carbon dioxide (CO₂) concentrations (Lal, 2004;
58 Hutchinson et al., 2007). Restoring SOC stocks provides numerous benefits including
59 improved soil and water quality, restoration of ecosystem services and increased agricultural
60 production (Lal, 2008). While the capacity for SOC sequestration is ultimately determined by
61 climate and local topography (Mackey et al., 2013) numerous agricultural management
62 practices have been recommended to facilitate SOC sequestration. These include: a) no-till or
63 reduced tillage; b) conversion of cropping soils to permanent pasture or incorporating pasture
64 phases into cropping rotations; c) retention of harvest residues or the addition of organic
65 matter (OM); d) adopting agroforestry; and e) nitrogen (N) fertilisation (Conant et al., 2001;
66 West and Post, 2002; McLauchlan, 2006; Powlson et al., 2011; Lorenz and Lal, 2014).

67 While N fertilisation can increase the input of fresh OM to soils by improving plant biomass
68 production (Buchanan and Cowan, 1990; Cowan et al., 1995), increasing N availability can
69 have varying effects on SOC stocks by affecting the decomposition of plant material, and the

70 formation and retention of SOM (Carreiro et al., 2000; Al-Kaisi et al., 2008; Frey et al.,
71 2014). Examples exist of N addition increasing SOC stocks (Gregorich et al., 1996; Alvarez,
72 2005; Dalal et al., 2011; Frey et al., 2014), having site-specific or no effects (Hobbie, 2005;
73 Page et al., 2013; Robertson et al., 2015) or negative effects (Khan et al., 2007; Rabbi et al.,
74 2014). Thus, further complications are involved in using N fertilisation as a management
75 strategy for SOC sequestration.

76 The factors involved in the stabilisation and turnover of SOC have been expertly reviewed
77 elsewhere (Sollins et al., 1996; Baldock and Skjemstad, 2000; von Lützow et al., 2007;
78 Schmidt et al., 2011; Dungait et al., 2012). Briefly, SOC stabilisation and turnover involves
79 complex interactions between: a) the relative recalcitrance of organic molecular structures; b)
80 molecular interactions between OM and inorganic substances, such as sorption to form
81 organo-mineral complexes, or with other organic molecules to form complexes via abiotic
82 condensation; c) soil physical properties such as soil aggregation which may limit the
83 physical accessibility of SOC by microbes; and d) the size, composition and metabolic
84 activity of the soil microbial community. Soil OC is considered the organic fraction of
85 biologically derived OC located within and on the surface of soil (Baldock and Skjemstad,
86 2000). The OC associated with the < 53 µm particle size fraction of soil, or silt+clay fraction,
87 represents a relatively slow-cycling C pool (Skjemstad et al., 2004; von Lützow et al., 2007).
88 Here we name this fraction “humus” OC (HOC). The HOC often represents the quantitative
89 majority of the SOC, and observable changes in HOC can be informative in predicting total
90 SOC changes over time (Six et al., 2002; Skjemstad et al., 2004). Pyrolysis-gas
91 chromatography/mass spectrometry and ¹³C nuclear magnetic resonance (¹³C-NMR)
92 spectroscopy have demonstrated that this C pool primarily consists of microbially-associated
93 C, either directly as microbial biomass and its products or as products of decomposition
94 (Grandy and Neff, 2008; Baldock et al., 2013). The molecular nature of the HOC fraction is

consistently microbially-derived, however, processes that affect soil microbial communities, such as those affected by N fertilisation, can alter the relative abundance of specific molecular structures in this fraction (Grandy and Neff, 2008). The quantity of HOC is dependent on the above-mentioned processes affecting SOC turnover (Gleixner, 2013).

Nitrogen addition directly affects the microbial community composition and metabolic activity, which can have long-term effects on SOC cycling (Fog, 1988; Carreiro et al., 2000; Fierer et al., 2012). A high C use efficiency (CUE) of the microbial community favours microbial biosynthesis as opposed to greater mineralisation of SOC to CO₂ with low CUE (Manzoni et al., 2012). Carbon use efficiency, in turn, is a consequence of cellular C:N stoichiometric requirements and the soil ecological stoichiometry of the system (Manzoni and Porporato, 2009; Manzoni et al., 2012; Zechmeister-Boltenstern et al., 2015). This reflects the interconnectedness of soil N and C cycling (Knicker, 2011). The link between CUE, plant litter quality (including molecular structure and C:N ratio of plant biomass) and SOM formation and stabilisation has been well illustrated as the microbial efficiency-matrix stabilisation (MEMS) framework (Cotrufo et al., 2013). Presumably N fertilisation improves SOM formation and retention by improving microbial CUE, dependent on plant litter quality.

In a previous study, we concluded that N addition had varying effects on plant material decomposition, dependent upon the initial C:N stoichiometry of the system (Finn et al., 2015). The objective of this study was to investigate and compare the importance of soil edaphic and plant variables on: A) the transformation and retention of plant-derived OM as HOC in four varying soils and plant litter from three species of varying C:N ratios; B) whether standardising the C:N ratio of plant litter with urea-N addition would equilibrate the retention of HOC between plant species; and C) whether urea-N addition would improve the retention of HOC in plant species of relatively high C:N ratio (Figure 1). We hypothesised that: A) HOC retention would be dependent upon soil type and plant species, with greater

HOC in litter with decreasing C:N ratio and in soils capable of limiting microbial physical accessibility to HOC; B) HOC retention would be independent of plant species once the C:N ratio was standardised; and C) HOC retention would be improved by urea-N addition due to increased CUE during decomposition. To do this, fresh OM of varying molecular recalcitrance was supplied as ^{13}C pulse-labelled wheat (*Triticum aestivum* L.), buffel grass (*Cenchrus ciliaris* L.) or lucerne (*Medicago sativa* L.), which are species common to three contrasting agricultural systems – a cereal, pasture and a legume component of mixed pastures, respectively. Residues were added to four agricultural soils of contrasting physico-chemical properties involved in SOC accessibility by microbes (variable values of sand, silt, clay, bulk density, C, N, phosphorous and metal contents). Finally, variable amount of N was added to wheat or buffel grass in the form of urea-N to achieve similar C:N ratios in all of the plant materials and simulate N fertilisation. Changes in HOC were monitored in a temperature and moisture-controlled laboratory incubation over 365-days.

2. Materials and methods

2.1 Soil and ^{13}C -labelled plant materials

The soils and plant materials used in this study have been described previously (Finn et al., 2015). Briefly, soil (0 – 10 cm depth) was sampled from four pasture sites in Australia: Rockies (Queensland): -26.89726 N, 148.88844 E; Akaringa (Queensland): -28.30264 N, 150.21728 E; Wimmera (Victoria): -36.672547 N, 142.288467 E; and Hamilton (Victoria): -37.839358 N, 142.086697 E. Bulk soil was thoroughly mixed, dried at 40°C and sieved to < 2 mm particle diameter. A range of physical and chemical properties were measured, including: total organic C (TOC), total N (TN), pH, EC, sand, silt, clay, bulk density and Colwell-extractable phosphorous (P) (Finn et al., 2015) (Table 1). In addition, acid citrate and oxalate

extractable iron (Fe) and aluminium (Al) were measured (Rayment and Lyons, 2010) and the abundance of a range of soil metals was also measured by lithium metaborate fusion with a Katanax K2 (SPEX Sample Prep, Metuchen, NJ, USA). Soil labile dissolved organic C (DOC) and heat-extracted C (HEC) were also measured (Sparling et al., 1998). Microbial biomass C (MBC) was estimated with the chloroform fumigation and extraction method (Vance et al., 1987). The production of the ^{13}C -pulse labelled buffel grass, wheat and lucerne has been described by Finn et al. (2015), as well as the analysis of the plant materials for C, ^{13}C and N by isotope ratio mass spectrometry (IRMS) and for relative abundance of C functional groups by solid-state ^{13}C -NMR spectroscopy. Plant C, N and C functional groups are given in Table 2.

2.2 Soil incubations

The soil incubations have been described by Finn et al. (2015). Briefly, ^{13}C -pulse labelled plant material was added to soils at the rate of 7.5 tonnes ha^{-1} . Urea-N was added to a final concentration that brought the amount of N being added to the soil (i.e. in plant material plus urea) to the same amount as that added by the lucerne material, resulting in urea-N additions of 242.6 mg N kg^{-1} soil to control soils, 113 mg N kg^{-1} soil to wheat treatments and 141.6 mg N kg^{-1} soil to buffel grass treatments. This was done to standardise the amount of N added relative to the lucerne treatment in order to better compare the treatments in terms of the total amount of N added to the system. This resulted in a total of seven treatments: (i) control soil only, (ii) control soil + N, (iii) soil + wheat, (iv) soil + wheat + N, (v) soil + buffel grass, (vi) soil + buffel grass + N, and (vii) soil + lucerne.

Soil (150 g oven dry equivalent mass) and plant material (excepting controls) were thoroughly mixed and wet up in a shallow tray using a spray bottle containing either

deionised water or a urea-N solution until the soil reached field capacity before transferring to a 250 mL plastic container. Field capacity was determined using the container capacity method, which is equivalent to 55 – 75% water-filled pore space (Cassel and Nielsen, 1986). The plastic containers containing the soils were placed in 1.5 L glass incubation jars. A total of three experimental replicates were prepared per treatment, with sufficient jars for destructive sampling on four occasions. Soils were incubated in the dark at 25°C. Open jars of water were placed in the incubator to humidify the atmosphere and reduce the rate of water loss. The weight of soils was measured weekly and deionised water added in case of water loss. On 30, 90, 180 and 365 days, soils were removed from incubation and oven-dried at 40°C for 48 hours.

2.3 HOC fractionation and IRMS analysis

Physical and chemical methods were used to extract SOC fractions from oven-dried soils at days 0, 30, 90, 180 and 365. Ten grams of soil treated with 30 mL of 5 g⁻¹ L sodium hexametaphosphate (ChemSupply, Australia) was physically fractionated with an automated vibratory sieve shaker (Fritsch Analysette 3, John Morris Scientific, Australia) system (Baldock et al., 2013). A 53 µm sieve separated the HOC fraction (< 53 µm particle diameter size) from the particulate organic C (POC) fraction (≥ 53 µm particle diameter size). Organic C (%) and the ¹³C isotope ratio (atom % abundance) were quantified from both fractions via direct combustion with an Isoprime Isotope Ratio Mass Spectrometer (GV Instruments, Manchester, UK) after the fractions were ground to a particle diameter size of < 100 µm.

2.4 Calculation of Δy -HOC (%)

All statistical analyses were performed in R v 3.2.2 (R Core Team, 2013). A weighted two-compartment mixing model was used to determine the mass of input plant ^{13}C (mg C g^{-1} soil) at each time point, for each plant species, in the HOC fraction (Equation 1) (Dalal et al., 2011).

$$y_t = x_t \cdot (z_t - w_t) / (A - w_t) \quad (\text{Eq.1})$$

Where x_t is OC (mg C g^{-1} soil) of the sample at time t , z_t is the ^{13}C atom % of the sample, w_t is the ^{13}C atom % of the control and A is the ^{13}C atom % of the insoluble plant fraction. The y_t (^{13}C mg C g^{-1} soil) was plotted over time for all treatments as an average of three experimental replicates. A two-compartment mixing model was used despite non-uniform labelling of soluble and insoluble plant material. This approach was chosen as plant soluble ^{13}C was selectively metabolised within the first 30 days of incubation, possibly within a week of incubation (Finn et al., 2015). Subsequently, plant soluble ^{13}C did not affect ^{13}C (mg C g^{-1} soil) over time. The proportional change in y was calculated as follows:

$$\Delta y = ((y_t - y_0) / y_0) \cdot 100 \quad (\text{Eq. 2})$$

Where y_0 is ^{13}C mg C g^{-1} soil at day 0 (t_0). Bartlett's test was applied to test for homogeneity of variance of Δy -HOC (%) after 365-days. Fisher's least significant difference (LSD) post hoc test with an α value of 0.05 was applied to Δy -HOC (%) across all treatment combinations (Mendiburu, 2015). A two-way analysis of variance (ANOVA) was used to test the effect of soil and litter interactions on Δy -HOC (%) after 365-days (Figure 1, Interaction A). Similarly, a two-way ANOVA was used to test the effect of soil and litter with standardised C:N ratio on Δy -HOC (%) after 365-days (Figure 1, Interaction B). Finally, a three-way ANOVA was used to test the effect of soil, litter and urea-N interactions on Δy -HOC (%) after 365-days (Figure 1, Interaction C).

214

215 *2.5 Boosted regression analysis of Δy -HOC (%)*

216 A gradient boosted machine (gbm) was used to model the effect of explanatory variables
217 listed in Table 3 on the Δy -HOC (%) (Natekin and Knoll, 2013; Ridgeway, 2015). Boosting
218 was chosen as the method sequentially builds an ensemble of regression trees that
219 successively improves the predictive accuracy of the overall ensemble at each iteration. This
220 results in an ensemble of trees that together offer better prediction than each tree would
221 separately. Analyses were performed separately for Interactions A, B and C. This was
222 performed with the ‘gbm’ package in R v 3.2.2 (Ridgeway, 2015). One thousand randomly
223 generated subsamples of half the scaled dataset were used to train the model, and the
224 corresponding test subsamples were used for cross-validation (James et al., 2013). The gbm
225 was optimised according to number of trees (1 – 5000) and interaction depth (1, 2, 4 and 8) to
226 avoid over-fitting the data. The optimal gbm was considered as the gbm with the least cross-
227 validated mean squared error (MSE). Once optimised, the gbm was used to compare the
228 relative influence of variables in explaining Δy -HOC (%). This procedure was carried out to
229 optimise gbms for analysis of Interactions A, B and C separately. Regression trees were
230 constructed to visualise how key variables described by the gbms could explain clustering of
231 Δy -HOC (%) for all interactions (James et al., 2013; Ripley, 2015).

232

233 **3. Results**

234 *3.1 Change in Δy -HOC (%) over time*

235 Proportional changes in labelled-HOC relative to 0 at t_0 are presented as Figure 2. Generally,
236 there was an increase in Δy -HOC (%) within the first 30-days of incubation, with greater

gains in buffel grass and wheat treatments. Δy -HOC (%) decreased after 30-days of incubation, although there was a net gain in labelled-HOC relative to t_0 with certain soils and plant materials (e.g. Rockies buffel and wheat treatments, Akaringa wheat treatments) compared to net losses (e.g. Wimmera buffel treatments, all lucerne treatments). The Δy -HOC (%) after 365-days of incubation varied greatly between treatments, as shown by a LSD post hoc test (Table 4). A two-way ANOVA of Interaction A confirmed significant influences of both soil and litter variables on Δy -HOC (%) ($P = < 0.001$) and a soil x litter interaction ($P = < 0.001$) (Table 4). In general, the Rockies soil demonstrated the largest and most consistent increases in Δy -HOC (%) whereas the Wimmera soils demonstrated losses. Δy -HOC (%) from wheat and buffel appeared to be soil dependent, whereas lucerne consistently resulted in losses. This was in support of hypothesis A as HOC was dependent upon soil and litter. A two-way ANOVA of Interaction B indicated significant influences of both soil and litter variables on Δy -HOC (%) ($P = < 0.001$ for each) and a soil x litter interaction ($P = < 0.001$). An effect of litter on HOC despite standardising the C:N ratio of the plant species with urea-N rejected hypothesis B. Finally, in a three-way ANOVA of Interaction C, soil and litter significantly influenced Δy -HOC (%) ($P = < 0.001$ and $P = 0.002$, respectively) whereas urea-N had no direct effect on Δy -HOC (%) ($P = 0.33$). This rejected hypothesis C, as urea-N addition alone had no effect on HOC. However, as soil x urea-N, litter x urea-N and soil x litter x urea-N also influenced Δy -HOC (%) ($P = < 0.001$ for each) this indicated that urea-N did have an effect which was soil and litter dependent. With the exception of Akaringa buffel and Wimmera wheat treatments, the effect of urea-N addition led to increased loss of Δy -HOC (%).

3.2 Boosted regression analysis of Δy -HOC (%)

To better explain the variability observed in Δy -HOC (%), the total dataset (Supplementary Table 1) was partitioned to compare the effects of soil and litter on Δy -HOC (%) (Interaction A), the effects of soil and litter with standardised C:N ratio on Δy -HOC (%) (Interaction B) and the effects of soil, litter and urea-N addition on Δy -HOC (%) (Interaction C). Three gbms were optimised to identify whether different explanatory variables were involved in Δy -HOC (%) under Interactions A, B and C. Cross-validated MSE of the gbms are given as Supplementary Figures 1 (Interaction A), 2 (Interaction B) and 3 (Interaction C). The relative influence of soil physico-chemical and plant molecular structural variables on Δy -HOC (%) was modelled using tree number of 500 and interaction depth of 8 for Interaction A; tree number of 275 and interaction depth of 1 for Interaction B; and tree number of 350 and interaction depth of 4 for Interaction C. All gbms predicted that soil edaphic variables had the greatest relative influence on Δy -HOC (%), shown as Figures 3 (Interaction A), 4 (Interaction B) and 5 (Interaction C). The key variables involved in Interaction A were TOC (35 ± 2.3 %), TN (18.9 ± 1.9), peak MBC (5.9 ± 1.3 %) and total C:N ratio (5.8 ± 0.8 %). Interaction B could almost be fully explained by DOC (41.9 ± 1.5 %), TOC (18.9 ± 2.2 %), peak MBC (8.4 ± 1.7 %) and sand (6.5 ± 1.8 %). Finally, the key variables involved in Interaction C were peak MBC (17.6 ± 1.3 %), sand (14.1 ± 2.4 %), TOC (13.8 ± 1.7 %) and total C:N ratio (9.1 ± 0.6 %). The effect of soil MBC at days 90 and 180 instead of day 30 were also compared (Supplementary Figure 4). The relative importance of soil MBC in predicting Δy -HOC (%) decreased by day 180.

3.3 Regression tree analysis of Δy -HOC (%)

Regression tree analysis was chosen to visualise positive versus negative relationships between explanatory variables identified by gbms and Δy -HOC (%) (Figure 6). Nodes

(numbered) indicate the values of explanatory variables which best describe splitting of sub-populations of Δy -HOC (%) clusters through recursive partitioning, represented as the mean of Δy -HOC (%) clusters at leaves (lettered). Of the four variables > 5 % relative influence explaining Interaction A, only soil TOC, TN and peak MBC were independent and significantly explained clustering of Δy -HOC (%) (Figure 6, A.). The greatest gain in Δy -HOC (%) occurred when soil TOC was less than 12.05 mg C g⁻¹ soil (Figure 6 A., leaf A). The greatest losses in Δy -HOC (%) occurred when TN was less than 3.45 mg N g⁻¹ soil and MBC was greater than 295.35 mg C kg⁻¹ soil (Figure 6 A., leaf D) and when TN, MBC and TOC were all relatively low (Figure 6 A., leaf B). Interestingly, in Interaction B, Δy -HOC (%) could be partitioned in half by DOC (Figure 6., B). It was more likely that gains in Δy -HOC (%) were observed in soils of less than 0.84 mg C g⁻¹ soil, particularly with high sand content (Figure 6 B., leaf C). Peak MBC best described losses of Δy -HOC (%) in Interaction B, although increasing MBC alone could not explain the greatest losses of Δy -HOC (%) as the largest losses occurred with an MBC between 357 – 426 mg C kg⁻¹ soil (Figure 6 B., leaf E). Regression tree analysis of Interaction C indicated that, in these treatments, only peak MBC and C:N ratio were required to independently and significantly explain clustering of Δy -HOC (%) (Figure 6., C). The greatest gains in Δy -HOC (%) occurred in soils of less than 205 mg C kg⁻¹ soil peak MBC (Figure 6 C., leaf A). Losses occurred in soils of greater than 388 mg C kg⁻¹ soil peak MBC (Figure 6 C., leaf E) and under conditions of relatively high MBC (> 295 mg C kg⁻¹ soil) with increasing C:N ratio (> 13.2) (Figure 6 C., leaf D).

4. Discussion

4.1 Ecological stoichiometry primarily explained Δy -HOC (%)

In this study we were interested in identifying the key variables that explained change in newly formed HOC and how N addition affected this process in a manner that gives rise to varied field observations (Hobbie, 2005; Khan et al., 2007; Page et al., 2013; Frey et al., 2014). ^{13}C (mg C g^{-1} soil) derived from ^{13}C -labelled plant material was monitored periodically over 365-days as a proportional change in the HOC fraction, $\Delta\text{y-HOC}$ (%). We focussed primarily on HOC as it forms the bulk of SOC bound by hydrophobic and organo-mineral interactions and can be considered as relatively slow cycling, sequestered C (von Lützow et al., 2007; Dungait et al., 2012). The three plant species utilised for decomposition in this study, buffel grass, wheat and lucerne, were chosen as they represent both agriculturally important species as well as varying in several aspects known to influence the transformation of plant-derived C into SOC. Finally, the four soils chosen were agriculturally relevant pasture soils of varying soil physico-chemical factors involved in stabilising and limiting microbial physical accessibility of SOC.

With these contrasting soils and plant substrates, $\Delta\text{y-HOC}$ (%) was principally driven by only a few soil edaphic variables in the three hypotheses considered here. These variables were the soil TOC, TN, MBC, DOC, C:N ratio and sand content. With the exception of sand content, these variables are all directly involved in describing how the ecological stoichiometry of the system affects soil microbial activity. Specifically, TOC and DOC are energy sources for microbial activity, while TN represents a nutrient source that constrains microbial growth and activity, the C:N ratio is a measurement of relative nutrient (N here) limitation and dictates net nutrient immobilisation versus mineralisation, and the MBC is a measurement of the size of the microbial biomass (Zechmeister-Boltenstern et al., 2015). This is consistent with the emerging view that SOC persistence is predominantly dependent on the soil ecosystem, and less dependent on the inherent chemical recalcitrance of the soil organic matter itself (Schmidt et al., 2011).

In the absence of urea-N addition and across litter of varying C:N ratio (Interaction A), HOC was dependent upon TOC, TN and MBC. The TOC and TN variables are measures of resource stoichiometry, which encompass substrates for microbial activity such as soluble monomers, organic acids, amino-acids, peptides, and complex organic material of plant and microbial origin (Kögel-Knabner, 2002; Haynes, 2005). The greatest increases in Δy -HOC (%) were when TOC was less than 12 mg C g⁻¹ soil. This suggests that a primary regulator of ¹³C HOC retention was the availability of pre-existing SOM, whereby a higher abundance of potential resources resulted in a more active microbial community and greater loss of input ¹³C. The catabolism of SOM by MBC follows Michaelis-Menten dynamics whereby decomposition rates are dependent upon two steps: first, substrate and decomposer (or extracellular enzyme) must meet, and secondly an enzyme-catalysed reaction occurs (Gleixner, 2015). Indeed, even this is an over-simplification of SOC turnover and the concentration of substrates of varying lability should also be considered (McDaniel et al., 2014; Glanville et al., 2016). Thus, either low substrate (TOC, TN, DOC, etc.) or MBC will necessarily reduce the rate of SOM turnover. Another potential contributing factor could be the C saturation hypothesis, which posits that the potential for silt+clay to stabilise C is limited, and thus greater stabilisation of input C can be expected in soils with a low pre-existing C content compared to soils with 'C saturated' silt+clay (Hassink, 1997).

Conversely, the greatest loss of ¹³C HOC in Interaction A occurred due to a combination of relatively high TOC, low TN and high MBC (Figure 6 A., leaf D). These losses are suggestive of the microbial nutrient mining hypothesis, as an increasingly large microbial biomass catabolises ¹³C HOC to acquire limited organic N. This hypothesis is often used to explain examples of overflow metabolism by microorganisms and poor CUE when a particular nutrient becomes limiting (Moorhead and Sinsabaugh, 2006; Craine et al., 2007).

Standardising the C:N ratio of litter did not remove a litter effect from Δy -HOC (%) as hypothesised (Interaction B), but it did alter the dynamics of HOC formation and retention. As with Interaction A, dynamics between substrate availability and the size of the MBC dictated gains and losses of ^{13}C HOC, however N availability became less important due to urea-N supplementation. Of note is that the concentration of labile DOC was particularly important in explaining whether ^{13}C HOC was retained or lost (Figure 6., B). All the treatments to the right of node 1 (i.e. $> 0.84 \text{ mg DOC g}^{-1} \text{ soil}$) included either lucerne litter or were Hamilton soils (Table 1). According to the MEMS framework, labile plant-derived OC is of greater importance than recalcitrant OC for the formation of long-term SOM, as determined by the rate of microbial biosynthesis of SOM precursors (Cotrufo et al., 2013). However, this proposal considers greater relativity of lability versus recalcitrance across plant species than was tested here. Here we show that under conditions of standardised litter C:N ratio, particularly labile plant litter and/or soils of increasing DOC and MBC results in losses of plant-derived C inputs.

Thirdly, we hypothesised that the addition of urea-N to buffel grass and wheat treatments would lead to increases in the formation and retention of ^{13}C HOC by improving CUE (Interaction C). The soil and litter-dependent effect of urea-N addition altered the dynamics of HOC formation and retention by increasing the relative importance of the size of the MBC for ^{13}C HOC. Specifically, ^{13}C HOC was best retained in soils of low MBC (Figure 6 C., leaf A). The response of the microbial community to N addition is complex, which has given rise to many hypotheses: a) N addition inhibits lignin degrading enzymes and/or microorganisms (Carreiro et al., 2000); b) N addition shifts the soil microbial ecology from an oligotrophic system to a copiotrophic system which increases the rate of decomposition of labile C (Fierer et al., 2012); and c) N addition reduces the rate of decomposition of SOC if soil N content limits the growth of microorganisms by improving the CUE of the microbial community

(Craine et al., 2007; Manzoni and Porporato, 2009). The primary dependence of the size of the MBC for ^{13}C HOC is most likely due to increased SOC decomposition in response to urea-N addition, in agreement with a shift of the microbial community to a copiotrophic state. This is well supported by soil metagenomic analyses across N gradients, which demonstrate a higher relative abundance of microbial genes involved in respiration (i.e. ubiquinone-cytochrome reductase and ATP synthase), DNA and RNA metabolism, and extracellular enzymes involved in C and N cycling (i.e. chitinase and N-acetylglucosaminidase) in soils of increasing TN (Fierer et al., 2012). Additionally, enzyme activity assays confirm these observations, for example the production of β -glucosidase and cellobiohydrolase increase when C and N are added to decomposing litter in soil (Allison and Vitousek, 2005; Sinsabaugh et al., 2005; Chen et al., 2014). However, it should be noted that TOC and MBC are highly co-linear in these soils and that TOC may also be controlling ^{13}C HOC retention here, either as an energy substrate for microbial activity or the stabilisation of C in the silt+clay fraction via the C saturation hypothesis.

In agreement with previous investigations demonstrating varied effects of SOC stocks to N addition between soils (Hobbie, 2005; Lu et al., 2011; Bejarano et al., 2014) there was no universal response to urea-N addition in the four soils considered here (Table 4, $P = 0.33$). The ANOVA, gbm and regression tree analyses all support a soil x urea-N effect (Table 4, $P = < 0.001$) which can be best described by the C:N ratio (Figure 6 C., node 3). Where the MBC was relatively low and/or the C:N ratio was low, modest increases in $\Delta y\text{-HOC}$ (%) occurred (Figure 6 C., leaves B and C) relative to losses (Figure 6 C., leaves D and E). This is suggestive of sufficient co-metabolism of C and N by the microbial biomass which is associated with an improved CUE (Manzoni and Porporato, 2009; Manzoni et al., 2012). Thus, N fertilisation as a means to improve SOC stocks will depend upon the size of the MBC and the C:N stoichiometry of the system.

407

408 *4.2 The importance of plant and other soil physico-chemical variables on Δy -HOC (%)*

409 In regard to other plant and soil variables considered in this study, it was particularly
410 interesting that plant molecular structural variables had little relative influence on Δy -HOC
411 (%). The abundance of plant methoxyl (0.9 – 4.5 %), alkyl (0.8 – 2.3 %), ketone (0 – 1.5 %)
412 and O-alkyl C (0 – 1.2 %) were the only plant variables involved in Δy -HOC (%). Methoxyl
413 and alkyl C structures are both associated with particularly labile OC, such as amino acids,
414 amino sugars and proteinaceous material (Mathers et al., 2007). Methoxyl C groups are also
415 present in non-labile lignin (Kögel-Knabner, 2002). However, given the low presence of aryl
416 and O-aryl C in the three plant species utilised (Table 2), high lignin abundance is unlikely.
417 Thus, the most important plant molecular structural variables for describing Δy -HOC (%)
418 were associated with labile OC, with greater losses in Δy -HOC (%) observed in alkyl and
419 methoxyl-rich lucerne treatments.

420 The relative influence of soil metals and texture was also investigated. Soil metal content was
421 measured as soil metals are constituents of minerals involved in protection of SOM by
422 adsorption to mineral surfaces or entrapment within mineral aggregates, or involved in
423 organo-mineral bonding via cations (Ca, Mn, Al and Fe in particular, Baldock and Skjemstad,
424 2000) at the mineral surface. Thus, soil metals can be both indirectly and directly correlated
425 with Δy -HOC (%). The relative influence of the most important metals for Δy -HOC (%) were
426 Ca (1.5 – 6.5 %), Si (2.3 – 4.8 %), Zn (0.8 – 2.3 %), Zr (0.4 – 2.3 %) and Al (0.2 – 1.8 %).
427 These elements are essential for forming orthosilicates and sheet aluminosilicates involved
428 in soil aggregate formation and protection of OC from microbial catabolism (Miral, 2008;
429 Dungait et al., 2012). The influence of texture on Δy -HOC (%) was also considered. Sand
430 content had a relatively high predictive capacity compared to other edaphic variables (3.4 –

14 %), while clay and silt content played minor roles (0 – 0.4 % and 1.4 – 5.3 %, respectively). The influence of sand may be because MBC (and presumably microbial activity) tends to be inversely correlated with sand content (Dalal, 1998). Iron-oxide rich sandy loam soils, such as the Rockies soil analysed here, are also among the most stably micro-aggregated soils, which can limit physical accessibility of SOM by microbes (Sollins et al., 1996). Furthermore, sand content is inversely correlated with TOC, which may support a role for the silt+clay C saturation hypothesis (Hassink, 1997). The minor roles of clay and silt were unexpected, as the surface area to volume ratio of clay and silt particles improves protection of SOC by providing a greater capacity for the formation of organo-mineral bonds than sand particles (Baldock and Skjemstad, 2000). Alternatively, the capacity for silt and clay to predict ^{13}C HOC may be improved by considering them as a single silt+clay variable, which would be the inverse of sand content.

It is worth noting that there are several limitations with *in vitro* studies such as this. For example, local climate is often the most important variable for SOC cycling, and can account for greater than 80% of the observed variability in SOC stocks (Allen et al., 2013). Climatic factors such as temperature variance, vapour-pressure deficit and rainfall frequency were not involved in this temperature and moisture-controlled *in vitro* incubation. Secondly, organic matter was added at a single time point, whereas continuous inputs can increase the rate of SOC turnover by influencing the size, composition and activity of the microbial community (Kuzakov, 2010). Finally, while the amount of plant residue added in this incubation study is similar to other *in vitro* studies (Wang et al., 2004), many others consider direct effects of N addition on native litter more representative of *in situ* litter quantities (Carreiro et al., 2000; Hobbie, 2005; Khan et al., 2007; Frey et al., 2013). Thus, here we show that under temperature and moisture-controlled conditions, and similar amounts of input organic matter,

soil ecological stoichiometry is of relatively greater importance to explain formation and retention of ^{13}C HOC.

Conclusions

By tracking the transformation of ^{13}C -labelled plant-derived OM to ^{13}C HOC, the relative importance of a range of plant molecular and soil physico-chemical variables for HOC formation and retention was assessed. Of primary importance was the soil ecological stoichiometry (specifically TOC, DOC, TN, MBC and C:N ratio), supporting a central role for SOC persistence due to ecosystem properties, as opposed to recalcitrance of organic matter. Secondly, while urea-N addition had no significant effect on $\Delta\gamma\text{-HOC}$ (%), N addition caused humus retention to be more dependent upon interactions between the size of the microbial biomass, C:N stoichiometry and DOC content. Consequently, we conclude that the effectiveness of using N fertilisation as a management practice for SOC sequestration is likely to depend on the ecological stoichiometry of the system. In future, the exploratory boosted regression analyses used here could perhaps be improved by more direct measurements involved in soil ecological stoichiometric interactions, such as considering specific forms of labile OC, amino-acids and peptides (Hill et al., 2012), extracellular enzyme activity (Allison and Vitousek, 2005) and the composition of the microbial community (Fierer et al., 2012).

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Figure legends

Figure 1: Graphical visualisation of the three interactions compared in this study, and the litter C:N ratios of the treatments: Interaction A) Soil x Litter; Interaction B) Soil x Litter with standardised C:N ratio; and Interaction C) Soil x Litter x urea-N addition.

Figure 2: Proportional change in Δy -HOC (%) over 365-days, with (●) and without (○) urea-N addition (mean $\pm$ standard deviation, n = 3).

Figure 3: The relative influence (%) of plant molecular structure and soil physico-chemical explanatory variables on Δy -HOC (%) for Interaction A) Soil x Litter. Values represent the mean of 1000 replicates of the gradient boosted machine (gbm). Error bars represent the standard deviation of 1000 replicates of the gbm.

Figure 4: The relative influence (%) of plant molecular structure and soil physico-chemical explanatory variables on Δy -HOC (%) for Interaction B) Soil x Litter with standardised C:N ratio. Values represent the mean of 1000 replicates of the gradient boosted machine (gbm). Error bars represent the standard deviation of 1000 replicates of the gbm.

Figure 5: The relative influence (%) of plant molecular structure and soil physico-chemical explanatory variables on Δy -HOC (%) for Interaction C) Soil x Litter x urea-N addition. Values represent the mean of 1000 replicates of the gradient boosted machine (gbm). Error bars represent the standard deviation of 1000 replicates of the gbm.

695

696 Figure 6: A) Regression tree of Δy -HOC (%) clustering to visualise the effect of key variables
697 involved in Soil x Litter interactions; B) Regression tree of Δy -HOC (%) clustering to
698 visualise the effect of key variables involved in Soil x Litter with standardised C:N ratio; and
699 C) Regression tree of Δy -HOC (%) clustering to visualise the effect of key variables involved
700 in Soil x Litter x urea-N addition. Nodes (numbered) indicate splitting based on the
701 explanatory variable (TOC mg C g⁻¹ soil, DOC mg C g⁻¹ soil, TN mg N g⁻¹ soil, peak MBC
702 mg C kg⁻¹ soil, C:N ratio, sand content %) and leaves (lettered) are the mean of Δy -HOC
703 clusters (in %).

704

Table 1: Physical and chemical properties of soils used in this study. Abbreviations are as follows: total organic carbon (TOC), total nitrogen (TN), microbial biomass C (MBC), dissolved organic C (DOC), Colwell-extractable phosphorous (P) and electrical conductivity (EC). Values are the averages $\pm$ standard deviation of three experimental replicates.

Site	Rockies	Akaringa	Wimmera	Hamilton
Soil classification*	Alfisol	Vertisol	Vertisol	Alfisol
Sand (%)	69.4	35.5	26.3	43.6
Silt (%)	10.9	15.6	13.1	31.5
Clay (%)	24.7	48.9	60.6	24.9
$\delta^{13}\text{C}$ (‰)	-25.9	-25.5	-30	-33
Colwell-P (mg kg ⁻¹ soil)	< 2	13.3	21.3	29.3
pH	6.4	8.3	8.2	5.2
EC (mS)	0.0	0.1	0.2	0.1
TOC (mg g ⁻¹ soil)	10.5 $\pm$ 0.9	10.3 $\pm$ 1.2	12.6 $\pm$ 0.5	41.7 $\pm$ 1.4
TN (mg g ⁻¹ soil)	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	1 $\pm$ 0.1	3.4 $\pm$ 0.1
MBC (mg C g ⁻¹ soil)	93 $\pm$ 14	166 $\pm$ 25	257 $\pm$ 44	386 $\pm$ 6
DOC (mg g ⁻¹ soil)	0.3 $\pm$ 0	0.1 $\pm$ 0	0.2 $\pm$ 0	1.5 $\pm$ 0
Treatment TOC (mg g ⁻¹ soil)				
Buffel grass	12.9 $\pm$ 1.2	12.3 $\pm$ 0.1	15.9 $\pm$ 0.9	44.5 $\pm$ 0.3
Buffel grass + N	12.9 $\pm$ 1.2	12.3 $\pm$ 0.1	15.9 $\pm$ 0.9	44.5 $\pm$ 0.3
Wheat	11.8 $\pm$ 0.1	14.6 $\pm$ 1.6	14.8 $\pm$ 0.1	45.1 $\pm$ 1.3
Wheat + N	11.8 $\pm$ 0.1	14.6 $\pm$ 1.6	14.8 $\pm$ 0.1	44.9 $\pm$ 1
Lucerne	12.1 $\pm$ 0.1	12.8 $\pm$ 0.6	15.0 $\pm$ 0.3	45.3 $\pm$ 2.4
Treatment TN (mg g ⁻¹ soil)				
Buffel grass	0.7 $\pm$ 0	0.7 $\pm$ 0	0.9 $\pm$ 0	3.5 $\pm$ 0
Buffel grass + N	0.8 $\pm$ 0	0.9 $\pm$ 0	1.1 $\pm$ 0	3.5 $\pm$ 0
Wheat	0.6 $\pm$ 0	0.7 $\pm$ 0.1	1.0 $\pm$ 0	3.6 $\pm$ 0.1
Wheat + N	0.8 $\pm$ 0	0.9 $\pm$ 0	1.1 $\pm$ 0	3.5 $\pm$ 0
Lucerne	0.8 $\pm$ 0	0.9 $\pm$ 0	1.1 $\pm$ 0	3.5 $\pm$ 0
Treatment C:N ratio				
Buffel grass	19.7 $\pm$ 1.5	18.1 $\pm$ 0.1	17.9 $\pm$ 0.9	12.8 $\pm$ 0.1
Buffel grass + N	15.9 $\pm$ 1.8	14.1 $\pm$ 0.6	14.1 $\pm$ 0.9	12.8 $\pm$ 0.1
Wheat	18.4 $\pm$ 1.3	20.2 $\pm$ 0.4	14.7 $\pm$ 0.5	12.6 $\pm$ 0.1
Wheat + N	14.6 $\pm$ 0.4	16.7 $\pm$ 2.4	13.2 $\pm$ 0.1	12.9 $\pm$ 0.2
Lucerne	14.9 $\pm$ 0.2	14.6 $\pm$ 0.2	13.3 $\pm$ 0.2	13.0 $\pm$ 0.7
Treatment DOC (mg g ⁻¹ soil)				
Buffel grass	0.75 $\pm$ 0.1	0.7 $\pm$ 0.1	0.64 $\pm$ 0.1	2.2 $\pm$ 0.3
Buffel grass + N	0.75 $\pm$ 0.1	0.61 $\pm$ 0.1	0.64 $\pm$ 0.1	2.2 $\pm$ 0.3
Wheat	0.77 $\pm$ 0	0.63 $\pm$ 0.1	0.67 $\pm$ 0.1	2.4 $\pm$ 0.1
Wheat + N	0.77 $\pm$ 0	0.63 $\pm$ 0.1	0.67 $\pm$ 0.1	2.2 $\pm$ 0.3
Lucerne	1.1 $\pm$ 0.1	0.9 $\pm$ 0.1	1 $\pm$ 0.1	2.3 $\pm$ 0.4

*United States Department of Agriculture (Soil survey staff, 1999).

Table 2: Plant C and N analysis, and solid-state ^{13}C -NMR functional group properties. Reproduced from Finn et al. (2015a).

	Plant		
	Buffel grass	Wheat	Lucerne
C and N analysis			
Total C (%)	38.2	37.6	40.6
Soluble C (%)	0.32	0.34	0.57
Insoluble C (%)	38	38.1	42.74
Total N (%)	1.13	1.6	3.6
Soluble N (%)	0.01	0.02	0.07
Insoluble N (%)	0.96	1.13	3.78
Total C:N	33.8	23.2	11.2
Soluble C:N	39.16	14.78	8.45
Insoluble C:N	39.45	33.67	11.31
Soluble ^{13}C atom %	2.9	1.49	2.13
Insoluble ^{13}C atom %	2.14	1.92	2.53
^{13}C -NMR spectroscopy (%)			
Alkyl C (0 - 45 ppm)	7.66	14.32	26.34
N-alkyl/methoxyl C (45 - 60 ppm)	5.84	4.76	8.92
O-alkyl C (60 - 90 ppm)	64.46	60.75	40.66
Di-O-alkyl C (90 - 110 ppm)	11.85	10.31	7.5
Aryl C (110 - 145 ppm)	2.81	1.33	4.88
O-aryl C (145 - 165 ppm)	1.32	0.58	0.54
Carboxyl C (165 - 190 ppm)	5.79	6.77	10.36
Ketone C (190 - 210 ppm)	0.26	1.18	0.81
Alkyl C :O-alkyl C	0.12	0.24	0.65

Table 3: Plant and soil physico-chemical explanatory variables used for analysis of Δy-HOC (%) with a gradient boosted machine.

Name	Summary
Plant molecular structure	
- Alkyl C	Terminal methyl, long-chain aliphatics, fatty acids, waxes, cutins, suberins, lipids, hemicelluloses, acetate, polymethylene (%)
- Methoxyl C	-OCH ₃ , N-alkyl, amino acids (%)
- O-Alkyl C	Carbohydrates, cellulose, hemicellulose, polysaccharides, alcohols, amino sugars (%)
- Di-O-Alkyl C	Anomeric C ₁ of cellulose, tannin and lignin components (%)
- Aryl C	Polyphenols, lignin and tannin components, C- and H-substituted aromatic C, olefinic C (%)
- O-Aryl C	Phenols, lignin and tannin components, suberin, O- and N-substituted aromatic C, olefinic C (%)
- Carboxyl C	Carboxylic acid, amid and ester C, organic acids (%)
- Ketone C	Carbonyl C groups, organic acids, ketone and aldehyde C (%)
Plant chemistry	
- Plant soluble C	Total C (%) in the soluble fraction of each plant species
- Plant soluble N	Total N (%) in the soluble fraction of each plant species
- Plant soluble C:N ratio	The ratio of C to N in the soluble fraction of each plant species
- Plant insoluble C	Total C (%) in the insoluble fraction of each plant species
- Plant insoluble N	Total N (%) in the insoluble fraction of each plant species
- Plant insoluble C:N ratio	The ratio of C to N in the insoluble fraction of each plant species
Soil physico-chemistry	
- TOC	Total organic C (mg g ⁻¹ soil) of native soil and added plant material
- TN	Total N (mg g ⁻¹ soil) of native soil, added plant material and with/without urea-N addition
- total C:N ratio	The ratio of TOC to TN of the treatment including soil, plant material and with/without urea-N addition
- peak microbial biomass C	Microbial biomass C (mg kg ⁻¹ soil) measured at day 30 of the incubation
- DOC	Dissolved organic C (mg g ⁻¹ soil) of native soil and added plant material
- HEC	Heat-extracted organic C (mg g ⁻¹ soil) of native soil and added plant material
- Colwell extracted P	0.5 M NaHCO ₃ extractable P (mg kg ⁻¹ soil)
- pH	Potential of hydrogen (pH units)
- EC	Electrical conductivity (mS m ⁻¹)
- Sand	Particle size between 0.02 and 2 mm (%)

- Silt	Particle size between 2 and 20 µm (%)
- Clay	Particle size less than 2 µm (%)
- Bulk Density	Oven dry (105°C) weight of soil extracted with a soil corer in relation to the core volume (Mg/m ³)
- Citrate extracted Fe and Al	Acid citrate extractable Fe (%) and Al (%)
- Oxalate extracted Fe and Al	Acid oxalate extractable Fe (%) and Al (%)
Metal abundance	
- Elements measured include:	Al (%), Ca (%), Cr (mg kg ⁻¹ soil), Fe (%), K (%), Mg (%), Mn (mg kg ⁻¹ soil), Na (%), P (%), Si (%), Ti (%), Zn (mg kg ⁻¹ soil), Zr (mg kg ⁻¹ soil)

Table 4: Plant-derived ^{13}C (mg C g^{-1} soil) in the HOC fraction at days 0 and 365 of incubation, and proportional change in ^{13}C (%) over 365-days. Values are the mean $\pm$ standard deviation ($n = 3$). Letters by $\Delta\text{y-HOC}$ (%) indicate LSD results with an α value of 0.05, with different letters indicating the $\Delta\text{y-HOC}$ (%) which differed from each other. F statistics and P values of two and three-way factorial ANOVA are also presented.

Soil	Treatment	HOC ^{13}C mg C g^{-1} soil		$\Delta\text{y-HOC}$ (%)
		Day 0	Day 365	
Rockies	Buffel	0.53 ± 0	0.63 ± 0.02	19.5 ± 4^a
	Buffel + N	0.53 ± 0	0.76 ± 0.01	45.2 ± 1.4^b
	Wheat	0.41 ± 0	0.68 ± 0.01	66.1 ± 1^c
	Wheat + N	0.41 ± 0	0.6 ± 0.03	45.3 ± 5^b
	Lucerne	0.89 ± 0.04	0.75 ± 0.05	-15 ± 3.7^{de}
Akaringa	Buffel	0.65 ± 0.01	0.56 ± 0	-13.6 ± 2.5^{fde}
	Buffel + N	0.65 ± 0.01	0.68 ± 0.05	5.2 ± 5.9^{gh}
	Wheat	0.5 ± 0.01	0.52 ± 0.02	15.5 ± 12.86^{hi}
	Wheat + N	0.5 ± 0.01	0.56 ± 0.03	11.3 ± 8.4^{agh}
	Lucerne	0.75 ± 0	0.72 ± 0	-4.6 ± 0.1^{if}
Wimmera	Buffel	0.82 ± 0.04	0.68 ± 0.03	-17 ± 5.1^{de}
	Buffel + N	0.82 ± 0.04	0.73 ± 0.07	-10.3 ± 5.6^{fd}
	Wheat	0.7 ± 0.06	0.6 ± 0.06	-14.2 ± 1.5^{fde}
	Wheat + N	0.7 ± 0.06	0.64 ± 0	15.2 ± 14.5^a
	Lucerne	0.96 ± 0.1	0.75 ± 0.05	-20.9 ± 4.3^e
Hamilton	Buffel	0.5 ± 0	0.57 ± 0	15 ± 0.1^a
	Buffel + N	0.5 ± 0	0.46 ± 0.03	-8.9 ± 5.2^{fd}
	Wheat	0.48 ± 0.08	0.55 ± 0.08	14.5 ± 3^{ag}
	Wheat + N	0.48 ± 0.08	0.49 ± 0.15	-45 ± 7.8^j
	Lucerne	0.82 ± 0.03	0.7 ± 0.05	-15.5 ± 6.6^{de}
ANOVA	Interaction A	Parameter	F statistic	P value
		Soil	145.42	$< 0.001^{***}$
		Litter	164.09	$< 0.001^{***}$
		Soil:Litter	57.82	$< 0.001^{***}$
	Interaction B	Soil	81.01	$< 0.001^{***}$
		Litter	40.36	$< 0.001^{***}$
		Soil:Litter	30.89	$< 0.001^{***}$
	Interaction C	Soil	181.12	$< 0.001^{***}$
		Litter	11.85	0.002^{**}
		Urea-N	0.98	0.33
		Soil:Litter	30.98	$< 0.001^{***}$
		Soil:Urea-N	48.57	$< 0.001^{***}$
		Plant:Urea-N	21.68	$< 0.001^{***}$
		Soil:Litter:Urea-N	17.93	$< 0.001^{***}$

Significance: '***' $P = 0.001$; '**' $P = 0.01$

Figure 1

Interaction A			Buffel	Wheat	Lucerne
Soil	⇌ Litter	C:N	33.8	23.2	11.2
Interaction B			Buffel	Wheat	Lucerne
Soil	⇌ Litter + urea-N	C:N	11.2	11.2	11.2
Interaction C			Buffel	Wheat	
+ or – urea-N		C:N – urea-N	33.8	23.2	
Soil	⇌ Litter	C:N + urea-N	11.2	11.2	

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6