

Effects of grassland management on plant C:N:P stoichiometry: implications for soil element cycling and storage

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Abstract. The functioning of human-managed grassland ecosystems strongly depends on how common management practices (e.g., animal grazing and the chronic addition of fertilizing materials to soils) interact to influence plant and soil element stoichiometry. Here we use data from a 22-yr-long grassland experiment to address whether and how plant element stoichiometry (i.e., carbon [C], nitrogen [N], phosphorus [P] ratios) might respond to (1) animal grazing, (2) agricultural liming (i.e., CaCO_3) applications, and (3) nutrient fertilization. We also ask whether plant C:N:P stoichiometry could predict changes in soil N and P availability and in soil C, N, and P stocks. We found that grassland management significantly affected plant C:N:P ratios as predicted by ecological stoichiometry theory. For example, plant aboveground and belowground C:N and C:P ratios decreased under chronic N and P fertilization, respectively. Plant C:N and C:P ratios were significantly greater in unfertilized (control) soils. Also plant C:N ratios were highest under P-only additions, whereas plant C:P ratios were highest under N-only additions. However, unpredictable changes in C:N:P ratios also occurred, suggesting that plant tissue chemistry may not be a simple reflection of soil nutrient availability. Changes in plant C:nutrient ratios well predicted variation in soil nutrient availability, but not in soil C, N, and P stocks. Contrary to expectations, soil C stocks significantly increased with decreasing plant C:N ratios in the nutrient-fertilized grasslands and not with increasing plant C:N ratios in the unfertilized grasslands. We suggest that a better mechanistic understanding of the negative relationship between plant C:N stoichiometry and soil C accrual will greatly help in improving the sustainability of human-managed grasslands.

Key words: grazing; liming; nutrient fertilization; plant C:N ratios; soil carbon sequestration; soil nitrogen availability; soil phosphorus availability.

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INTRODUCTION

Managed grassland ecosystems deliver key services to human society, including the production of forage and animal biomass, the regulation of water and nutrients, and the sequestration of atmospheric CO_2 in soils (Lavorel et al. 2011, Sousana and Lemaire 2014, Rumpel et al. 2015, Fornara et al. 2016). To maintain high productivity, grasslands are subject to multiple management

practices such as animal grazing and the addition of fertilizing materials to soils, which include both nutrients (e.g., N, P) and non-nutrients (e.g., agricultural lime, CaCO_3 ; Conant et al. 2001, Jones and Donnelly 2004, Leifeld 2012). While these practices enhance plant and animal biomass production, management intensification (e.g., repeated inorganic fertilization, heavy grazing) can have detrimental effects on the long-term sustainability of grassland ecosystems (McLauchlan 2006).

Management practices strongly affect the quality and quantity (i.e., tissue chemistry and total biomass) of plant detritus returned to the soil from both above- and belowground compartments (i.e., shoots and roots). This in turn can significantly influence soil biogeochemical properties and thus soils' ability to store and cycle C and N (Bellamy et al. 2005, Quinton et al. 2010, Cenini et al. 2015). Key knowledge gaps remain about how long-term grassland management might influence plant tissue chemistry, and whether or not changes in plant element stoichiometry (e.g., C:N:P ratios) might be related to predictable changes in soil C, N, and P content (%) and storage (i.e., soil C, N, and P pools). Previous studies show how animal grazing by enhancing N availability in soils (through dung and urine deposition) can significantly decrease plant above- and belowground C:N ratios while increasing plant belowground N:P ratios (Bai et al. 2012, Zheng et al. 2012). Experimental evidence also suggests that long-term additions of inorganic N forms to grassland soils will significantly reduce plant belowground C:N ratios (Gill et al. 2006, Fornara and Tilman 2012, Kirkby et al. 2013). Moreover, liming applications (e.g., repeated additions of CaCO_3) tend to influence N uptake by plants and thus affect plant tissue chemistry (Kemmitt et al. 2006) through liming-induced effects on soil pH, soil microbial activities, and rates of soil N mineralization (Rangel-Castro et al. 2004, Fuentes et al. 2006, Kemmitt et al. 2006). A better understanding of how grazing, nutrient fertilization, and liming applications might interact to affect plant stoichiometry, and in turn, soil processes can greatly help in improving the sustainability of managed grassland ecosystems.

From a soil ecosystem-functioning perspective, it is clear that common management practices such as grazing, nutrient, and liming applications tend (either directly or indirectly) to increase the availability of reactive forms of soil nutrients (e.g., nitrate levels). Thus, we might expect an increase in the relative abundance of N (and P) in plant tissues, and a consequent decrease in plant C:N and C:P ratios under long-term management. Chronically fertilized and grazed grasslands (on nutrient-rich soils) are associated with high productivity, fast plant growth rates, and low plant nutrient-use efficiency, which in turn should be linked to fast decomposition rates and soil

nutrient cycling but also to low levels of soil C accumulation (Hobbie 1992, Sterner and Elser 2002, Wardle et al. 2004). On the other side, unfertilized and ungrazed grasslands (on nutrient-poorer soils) are associated with plant species, which have higher C:N tissue ratios and higher nutrient-use efficiency resulting in slower decomposition rates and soil nutrient cycling and in higher levels of soil C accumulation (Hobbie 1992, Wardle et al. 2004). Overall, the direction and magnitude of these ecosystem processes will depend on complex interactions between plant traits, rates of litter decomposition, and intrinsic soil fertility levels, which vary across soil nutrient gradients in natural and semi-natural systems (Hobbie 2015). Nevertheless, in human-managed systems, we expect that long-term repeated additions of nutrient fertilizers and animal grazing will predictably (and differently) affect plant C:N stoichiometry and thus soil C and N stocks when compared to historically unfertilized and ungrazed grasslands.

Here we first addressed how 22 yr of common management practices (grazing, liming, and nutrient fertilization) have affected the relative abundance (i.e., stoichiometry) of different elements (C, N and P) in plant tissues both aboveground and belowground. Secondly, we addressed whether changes in plant element stoichiometry might be related to predictable changes in soil nutrient availability (N and P) and in total soil C, N, and P pools. This study aimed to improve our mechanistic understanding of how repeated grazing, liming, and nutrient fertilization might influence plant C:N:P ratios, and whether changes in plant stoichiometry might relate to changes in soil element cycling and pools. We specifically tested the following hypotheses:

1. Chronic animal grazing by rabbits (*Oryctolagus cuniculus*) will contribute to decreased plant C:N and C:P ratios, but increased plant N:P ratios.
2. Long-term additions of agricultural lime (i.e., CaCO_3) will decrease plant C:N ratios mainly because of positive liming-induced effects on soil N availability.
3. Nutrient additions will affect plant C:N:P ratios in predictable ways as suggested by ecological stoichiometry theory (Sterner and Elser 2002):

- a. The C:X ratio ($X = \text{N or P}$) of plant tissues increases when the availability of nutrient X in soils decreases;
 - b. The C:X ratio ($X = \text{N or P}$) of plant tissues increases with the decreased availability of X relative to another soil limiting resource (i.e., we expect C:N ratios to increase when P:N ratios also increase).
4. Grassland communities, which have received repeated N additions for >20 yr, will be associated with lower plant C:N ratios, higher soil N cycling (i.e., greater soil N availability), and smaller soil C stocks when compared with unfertilized grassland communities.

MATERIALS AND METHODS

Study site

Nash's Field is a long-term grassland experiment located at Silwood Park, Berkshire, UK. The site is located on the freely draining acidic and sandy soils of the Bagshot series, which are classified within the Eutric Cambisol group (FAO 1998). This grassland was historically managed as a hay meadow until 1991 when a four-factorial split plot design experiment was established. Specifically, four randomly placed blocks ($22 \times 44 \text{ m}$) were initially split to control for invertebrate herbivory (\pm insects). Each block was split into two plots to control for vertebrate herbivory (\pm rabbits), each herbivory plot was split to control for soil pH (\pm agricultural liming), and finally, each liming plot was split to control for different nutrient additions (N, P, K and Mg) in various combinations (see Appendix S1: Fig. S1; also see <http://www.imperial.ac.uk/silwood-park/research/silwood-lte/nashk-s/>). The smallest experimental plots (i.e., nutrient plots) measure $2 \times 2 \text{ m}$ each (Crawley 1990). Thus, 4 blocks \times 2 rabbit grazing treatments \times 2 liming treatments \times 5 nutrient treatments = 80 individual experimental units. The five nutrient treatments are N-only, P-only, N + P + Mg, all nutrients (N + P + K + Mg), and no nutrient (i.e., control plots). A previous study of the same long-term grassland experiment shows how aboveground plant biomass production in plots fertilized with all nutrients (i.e., NPKMg) is more than twice (roughly 7 Mg of dry mass per ha) the plant biomass production of control plots (3 Mg dry mass per ha; Fornara et al. 2013). However, this biomass

is harvested (i.e., grass cut) every year and removed from these plots (Crawley 1990) with little C inputs thus returned to the soil from aboveground.

Mineral nutrients are added to the soil each year as 100 kg N/ha in the form of ammonium nitrate (NH_4NO_3), 35 kg P/ha in the form of triple superphosphate, 225 kg K/ha in the form of muriate of potash, and 11 kg Mg/ha in the form of Epsom salts. Thus, 4 randomly located large plots \times 2 grazing treatments (\pm rabbits) \times 2 liming treatments (\pm lime) \times 5 nutrient treatments (control, All, N-only, P-only, and NPMg) = 80 experimental plots sampled in our study. A previous study shows how 73% of total plant aboveground biomass in these experimental plots is produced by C3 grasses regardless of nutrient fertilization treatment, whereas non-leguminous forbs contribute between 10% and 16% and legumes <0.1% of plant community biomass (Fornara et al. 2013).

Soil sampling and analyses

Soil samples were collected in May 2014 at four locations within each of the 80 experimental plots using a 2.5-cm soil corer to a depth of 20 cm. Following initial collection, soils were pooled and sieved through a metal mesh (2 mm mesh size) to remove roots and any other detritus before soils were dried at 70°C for 3 d. A dried soil subsample was ground and analyzed for total C and N (%) content by combustion and gas chromatography using a COSTECH Analytical ECA 4010 instrument (Costech Analytical Technologies Inc., Valencia, California, USA). A further subsample was used to measure soil pH via the creation of a 1:5 soil:deionized water suspension. Fresh soil sub-samples were sieved through a 2 mm mesh size and analyzed for available phosphate using the Olsen P method (Olsen et al. 1982). An additional soil sample was collected from the center of each plot using a 4.3-cm corer to a depth of 20 cm in order to calculate soil bulk density (i.e., mass of dried soil per unit volume of soil collected).

Plant aboveground and belowground sampling and analyses

Plant aboveground biomass samples from each of the 80 experimental plots were collected over 2 d in June 2014. A $25 \times 25 \text{ cm}$ PVC frame quadrat was placed at the center of each plot, and all aboveground plant biomass inside the quadrat was clipped to ground level, harvested, dried at

70°C for 3 d, and then ground to be analyzed for total C, N, and P content. Total C and N were determined by combustion using a 1500 NA Carlo-Erba element analyzer (Elan, Lakewood, New Jersey, USA). Total P in plant material was analyzed using a two-step protocol; first, organic phosphorus and polyphosphates were converted to ortho-phosphate, and secondly, ortho-phosphate was determined based on a colorimetric method (Murphy and Riley 1962). In May 2014, three soil cores (2.5 cm in diameter and 20 cm deep) from each plot were collected to estimate total plant belowground biomass (i.e., total root mass). Samples were homogenized and washed over a 2-mm-mesh-size sieve with water to remove soil and any detritus. Once washed, roots were dried at 60°C for 3 d before being weighed, ground, and analyzed for total root C, N, and P content.

Potential net soil N mineralization rates

Six soil samples (0–20 cm soil depth) were randomly collected from each plot in June 2014 using a 2.5-cm soil corer. Soils were sieved through a 2 mm mesh size, stored in plastic bags in a cooler box for 24 h, and then processed in the laboratory. We first added 50 mL of 1 mol/L KCl solution to a 20-g soil sample collected from each plot, and then the solution was stirred to homogenize the soil and stored overnight at 4°C to allow the soil to settle. The following morning, 8 mL of the top, clear solution was transferred into plastic tubes and frozen to measure the concentration of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ using a Bran-Luebbe AA3 autoanalyzer (SEAL Analytical Ltd., Southampton, UK).

A further 20-g soil sample from each plot was incubated at room temperature for 30 d. Sufficient water was added to each sample after 2 weeks during the laboratory incubation to keep moisture constant. Soil samples were then processed following the same method to measure $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations after the 30-d incubation period. Net soil N mineralization rates were calculated by subtracting the initial 24-h concentrations of $\text{NH}_4^+\text{-N}$ and $\text{NH}_3^-\text{-N}$ from the 30-d concentrations.

Data analysis

The Nash's Field experiment has a split-split-split plot experimental design where nutrient addition, liming, and grazing treatments are nested

within each other and then replicated across four randomly located large plots (i.e., blocks; two of which have received a pesticide treatment and two of which have not; Appendix S1: Fig. S1). We thus used a mixed-effects ANOVA model where the four large blocks were included as random factors, and key experimental treatments (i.e., insects, grazing, liming and nutrient application) were included as fixed effects. We tested these potential effects on plant stoichiometry and different plant and soil parameters (i.e., above- and belowground C:N:P stoichiometry, plant biomass, soil C%, soil N%). Since the insect treatment did not account for any significant effect on the response variables, we produced simpler models which included grazing, liming, and nutrient fertilization. Final models were produced using the restricted maximum likelihood (REML) method. We also performed simple and multiple regression analyses to search for potential relationships between different plant and soil variables. Where appropriate, data were square-root-transformed prior to analysis in order to improve data distribution around the mean. All analyses were conducted using JMP version 11.2.1 statistical software (SAS Institute 2010).

RESULTS

Grazing had a significant negative effect on the C:N ratio of plant aboveground ($F_{1,60} = 37.5$; $P < 0.0001$) and belowground ($F_{1,60} = 7.06$; $P = 0.01$) compartments (Fig. 1a). Grazing also significantly negatively affected C:P ratios of plant aboveground ($F_{1,60} = 17.05$; $P = 0.0001$) and belowground compartments ($F_{1,60} = 4.37$; $P = 0.04$; Fig. 1b). Grazing had no effects, however, on the N:P ratio of plant aboveground ($F_{1,60} = 0.6$; $P = 0.45$) or belowground ($F_{1,60} = 0.06$; $P = 0.81$) compartments (Fig. 1c). Grazing significantly contributed to increased N (%) content of both belowground ($F_{1,60} = 16.6$; $P = 0.0001$) and aboveground plant compartments ($F_{1,60} = 29.9$; $P < 0.0001$), but not plant C (%) concentration ($P = 0.56$). Plant aboveground ($F_{1,60} = 15.4$; $P = 0.0002$) and belowground ($F_{1,60} = 9.9$; $P = 0.002$) P (%) content also increased under grazing.

Liming significantly increased soil pH ($P < 0.0001$; Appendix S1: Fig. S2) whereby limed plots had on average soil pH of 6.6 ± 0.05 , whereas pH of unlimed soils was 4.56 ± 0.05 . Liming had a significant positive effect on plant aboveground

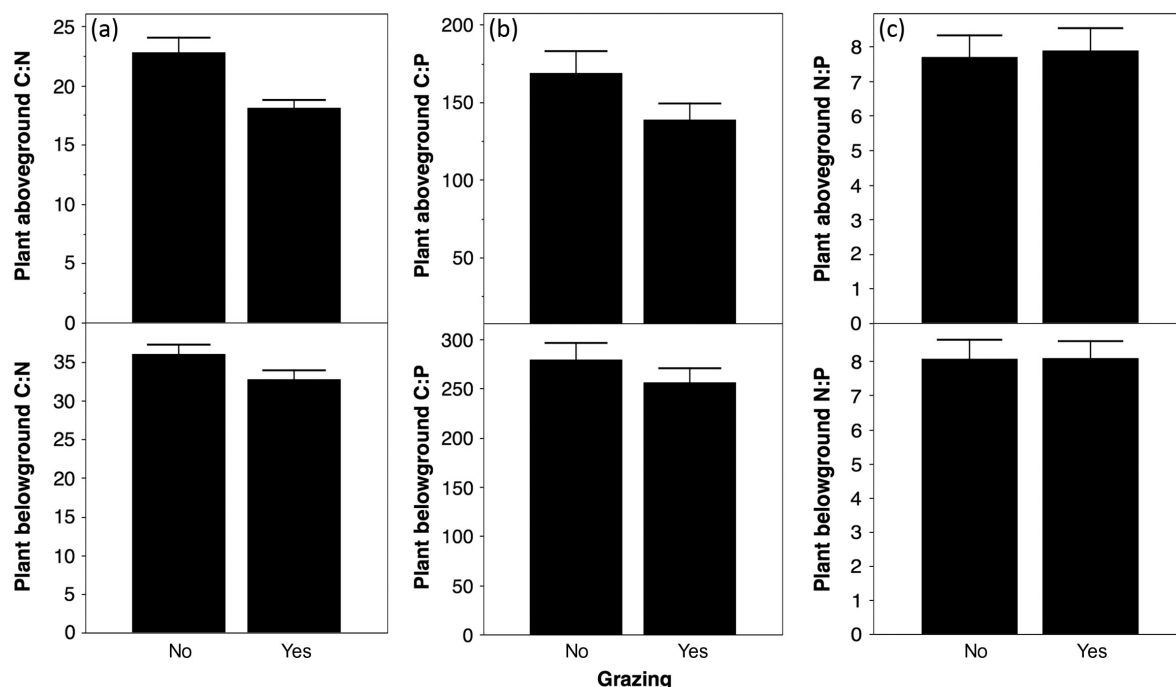


Fig. 1. Effects of animal grazing on (a) plant aboveground and belowground C:N ratios, on (b) plant aboveground and belowground C:P ratios, and on (c) plant aboveground and belowground N:P ratios. Error bars show variation between plots receiving the same grazing treatment.

C:N ratios ($F_{1,60} = 13.3$; $P = 0.0005$) but no effect on plant belowground C:N ratios ($F_{1,60} = 0.15$; $P = 0.70$; Fig. 2a). Liming also had a negative effect on plant belowground C:P ratios ($F_{1,60} = 37.4$; $P < 0.0001$; Fig. 2b) but had no effect on plant aboveground C:P ratios ($F_{1,60} = 0.44$; $P = 0.51$). Liming had a negative effect on both plant belowground N:P ($F_{1,60} = 38.41$; $P < 0.0001$) and plant aboveground N:P ratios ($F_{1,60} = 4.91$; $P = 0.031$; Fig. 2c). Liming contributed to a significant decrease in N (%) content aboveground ($F_{1,60} = 19.9$; $P < 0.0001$), but not belowground ($F_{1,60} = 2.3$; $P = 0.13$). Similarly, liming decreased plant aboveground C (%) content ($P = 0.0002$), but not plant belowground C (%) content ($P = 0.16$). Liming also led to a significant decrease in aboveground P (%) content ($F_{1,60} = 10.3$; $P = 0.002$), but a significant increase in belowground P (%) content ($F_{1,60} = 36.1$; $P = 0.002$).

Nutrient additions significantly affected N (%) and P (%) content of both plant above- and belowground compartments (see Appendix S1: Figs. S3, S4). Plant N (%) concentration in both aboveground and belowground compartments

was significantly higher when N was added either alone or in combination with other nutrients (Appendix S1: Fig. S3). Similarly, plant P (%) concentration in both aboveground and belowground compartments was significantly higher when P was added either alone or in combination with other nutrients (Appendix S1: Fig. S4). N additions either alone or in combination with other nutrients significantly increased plant aboveground C (%) content ($P = 0.005$), but not plant belowground C (%) content.

The addition of N (either alone or in combination with other key nutrients) had significant negative effects on plant above ($F_{4,60} = 41$; $P < 0.0001$) and belowground ($F_{4,60} = 17.2$; $P < 0.0001$) C:N ratios (Fig. 3a). The addition of N-only, and the absence of any nutrient fertilization (control treatment), had strong positive effects on plant aboveground ($F_{4,60} = 97$; $P < 0.0001$) and belowground C:P ratios ($F_{4,60} = 56.7$; $P < 0.0001$) (Fig. 3b). The addition of N-only had a strong positive effect on plant aboveground ($F_{4,60} = 158$; $P < 0.0001$) and belowground N:P ($F_{4,60} = 68.2$; $P < 0.0001$) ratios (Fig. 3c).

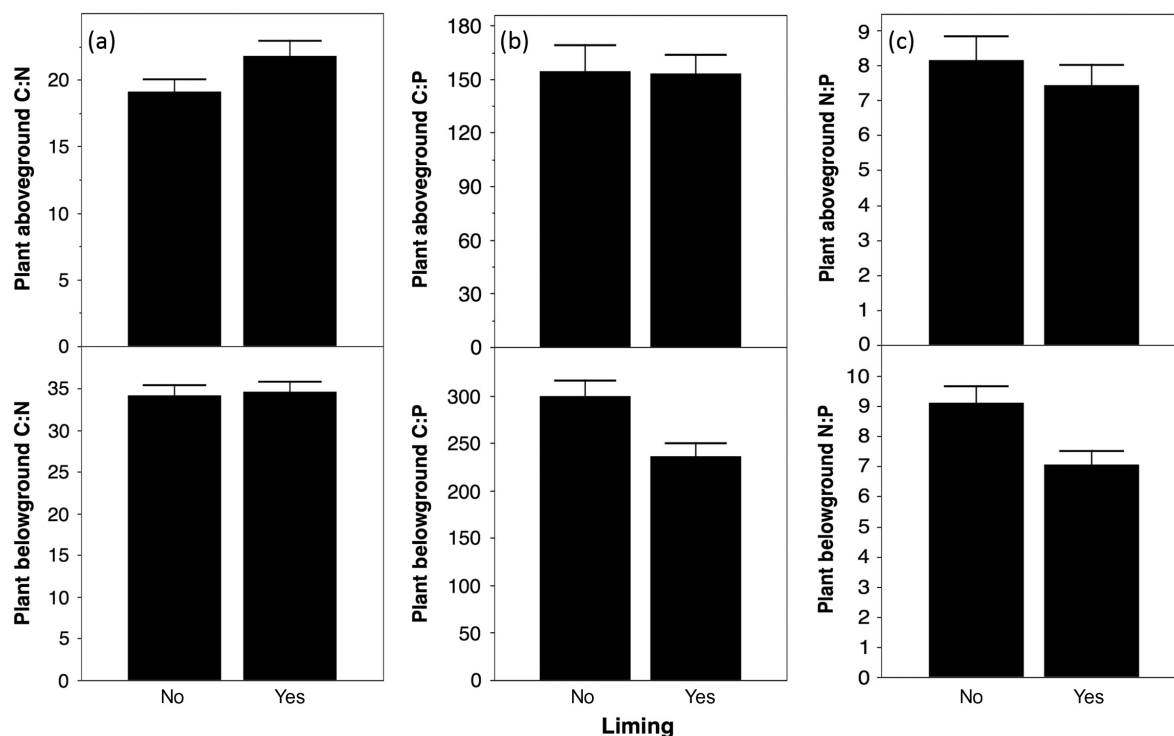


Fig. 2. Effects of agricultural liming on (a) plant aboveground and belowground C:N ratios, on (b) plant aboveground and belowground C:P ratios, and on (c) plant aboveground and belowground N:P ratios. Error bars show variation between plots receiving the same liming treatment.

Plant element stoichiometry and soil N and P availability

Results from regression analyses show that plant aboveground C:N ratios are negatively related to net soil N mineralization rates ($P = 0.001$; Fig. 4a) as well as plant belowground C:N ratios ($P = 0.0005$; Fig. 4b). Soil Olsen P (an index of P availability in soils) was significantly negatively related to both plant aboveground and belowground C:P ratios ($P < 0.0001$; Fig. 4c, d). We also found that soil P:N ratios were not significantly related to plant C:N ratios (Fig. 5a, b). However, soil N:P ratios were significantly positively associated with increases in plant belowground ($P < 0.0001$) and aboveground C:P ratios ($P < 0.0001$; Fig. 5c, d).

Plant C:N stoichiometry and soil C, N, and P content and stocks

We found a significant negative relationship between soil C (%) and both plant aboveground C:N and belowground C:N ratios (Fig. 6a, b). We

also found a significant negative relationship between soil C stocks (t C/ha) and plant aboveground ($P = 0.041$) and belowground ($P = 0.003$) C:N ratios (Fig. 6c, d). Soil C stocks were significantly higher under N-only and NPMg nutrient treatments, whereas unfertilized control plots showed the lowest soil C stocks (although not significantly different from All and P nutrient treatments; Appendix S1: Fig. S5). Soil C stocks (t C/ha; $P < 0.0001$) were significantly positively related to soil N (%) content (Appendix S1: Fig. S6). We found a significant negative relationship between soil N (%) and plant belowground ($P = 0.002$) and aboveground C:N ratios ($P = 0.003$). Soil N stocks (t N/ha) were not related to plant aboveground ($P = 0.20$) or belowground C:N ratios ($P = 0.054$). There was a significant negative relationship between soil P (%) and both plant belowground ($P < 0.0001$) and aboveground C:P ratios ($P < 0.0001$). Finally, soil P stocks were negatively related to plant aboveground ($P = 0.0002$) and belowground C:P ($P = 0.002$) ratios.

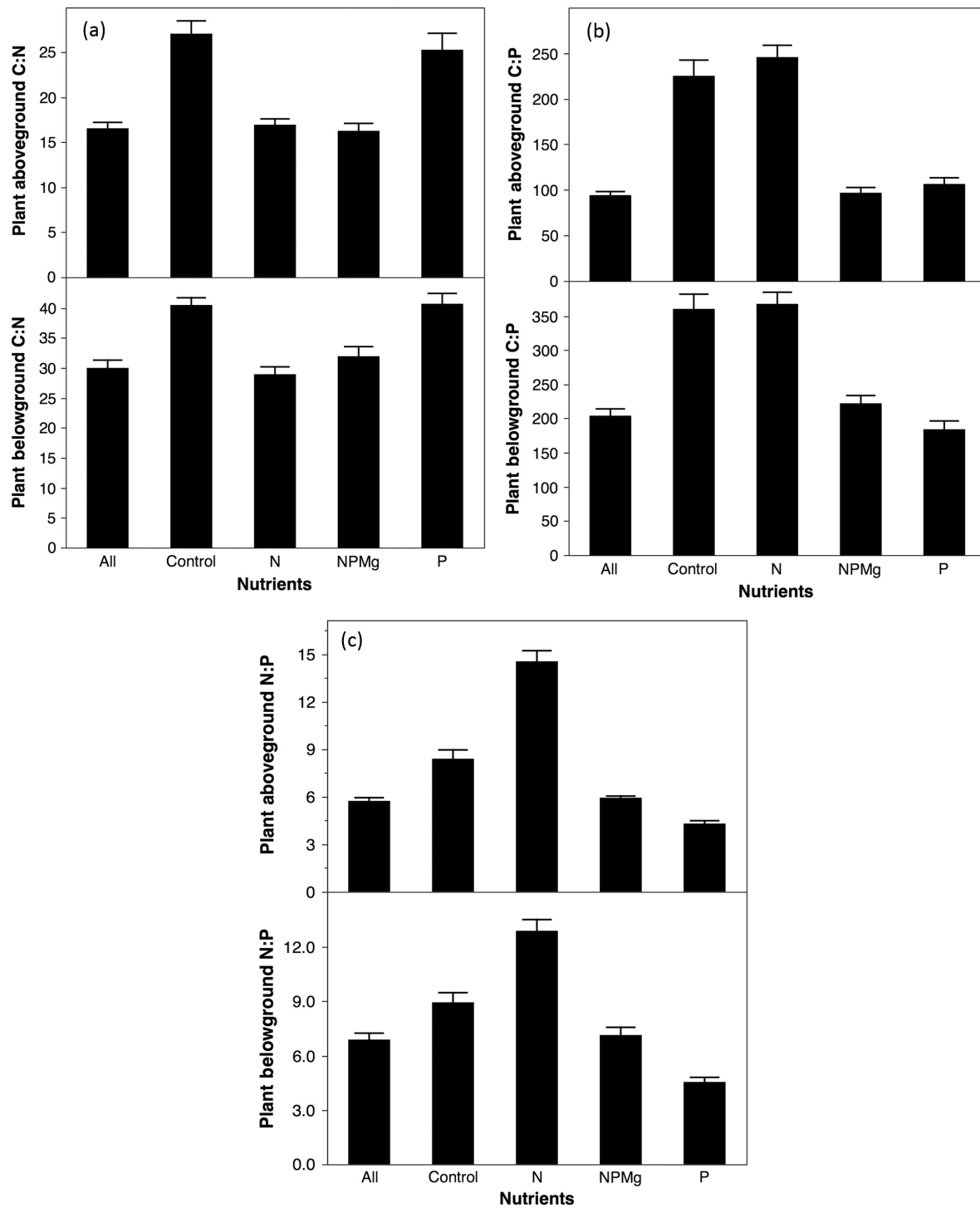


Fig. 3. Effects of nutrient fertilization on (a) plant aboveground and belowground C:N ratios, on (b) plant aboveground and belowground C:P ratios, and on (c) plant aboveground and belowground N:P ratios. Error bars show variation between plots receiving the same nutrient treatment.

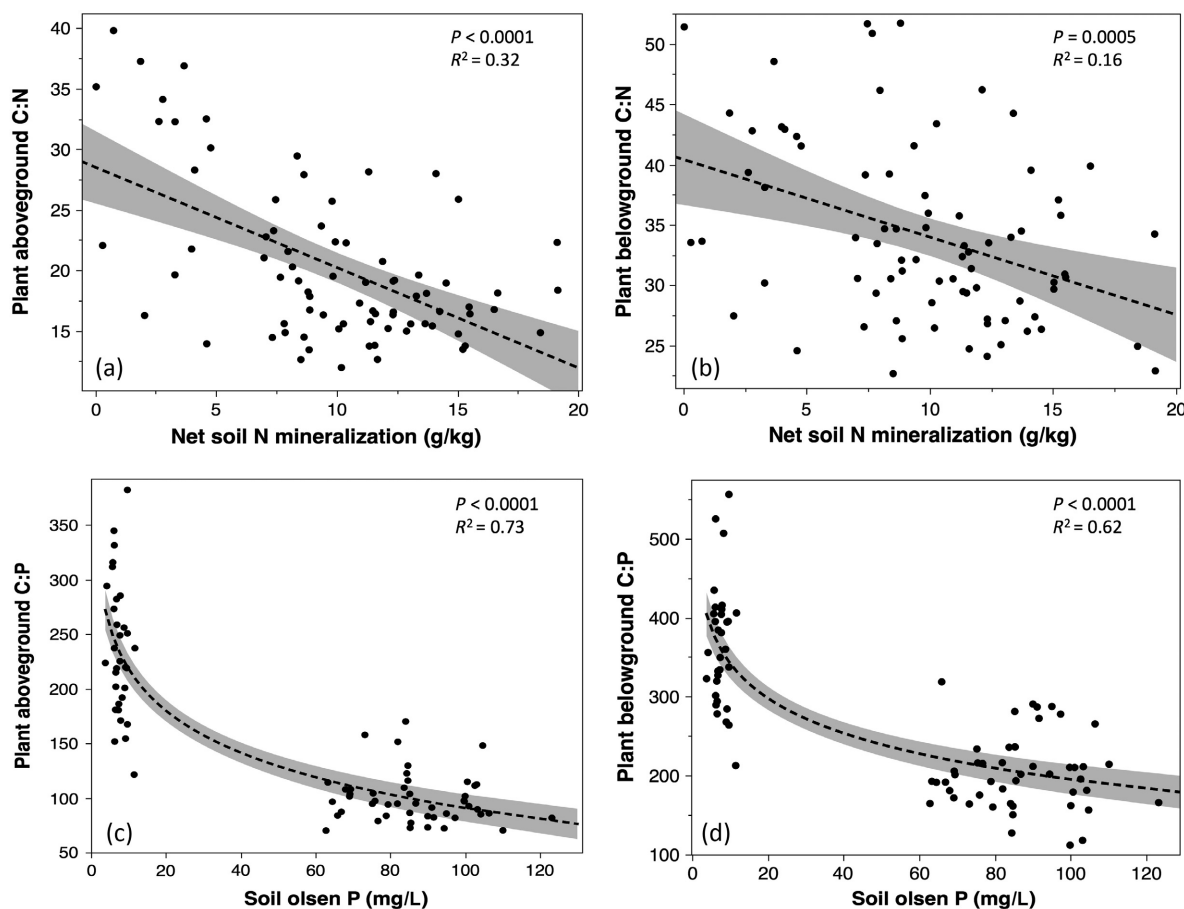


Fig. 4. Relationships between net soil N mineralization rates and (a) plant aboveground and (b) belowground C:N ratios. Also relationships between soil Olsen P and (c) plant aboveground, and (d) belowground C:P ratios. Gray shaded areas indicate 95% confidence interval limits.

DISCUSSION

Overall, our results show that long-term grassland management has significantly affected both plant aboveground and belowground C:N:P stoichiometry. We found that most of plant element ratio responses to grazing, liming, and nutrient additions could be predicted by ecological stoichiometry theory. However, unpredictable changes in C:N:P ratios also occurred, suggesting that plant tissue chemistry may not be a simple reflection of soil nutrient availability. A key finding of our study is that relationships between plant C:N ratios and soil C stocks were weak or opposite to those generally expected from plant-herbivore-soil interactions when comparing nutrient-poor *vs.* nutrient-rich ecosystems. For example, we would

expect that aboveground-belowground interactions within fertile, more productive grasslands will lead to lower soil C accumulation than in infertile, less productive grasslands (see Wardle et al. 2004). Also we would expect that stoichiometrically imbalanced (Stern and Elser 2002) grasslands, which have higher plant C:N ratios and higher plant N-use efficiency, would accumulate more C in soils when compared with stoichiometrically balanced grassland systems, which have lower plant C:N ratios and lower plant N-use efficiency. Opposite to these predictions, we found that soil C content and soil C stocks increased under decreasing plant C:N ratios (Fig. 6) in grassland communities, where soil C stocks are particularly high under long-term N-only additions (Appendix S1: Fig. S5).

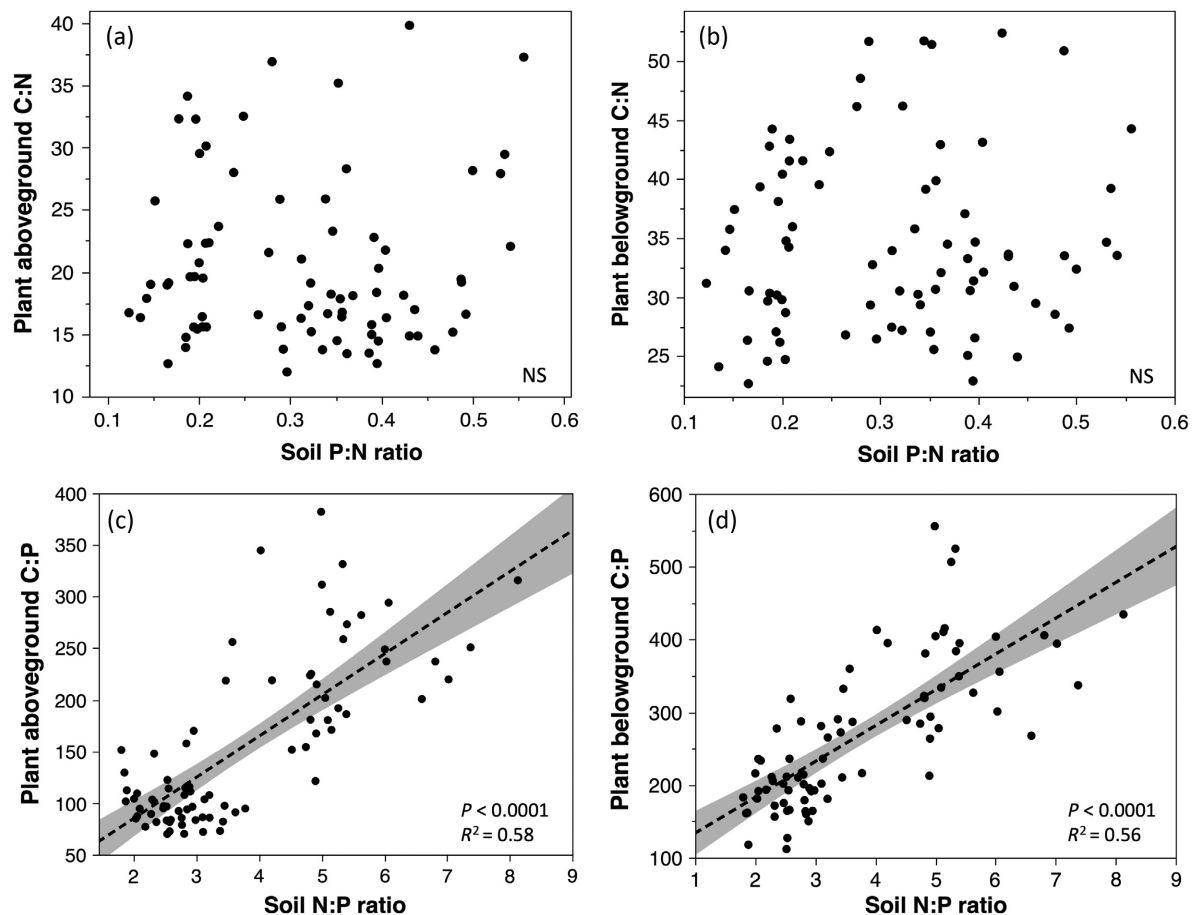


Fig. 5. Relationships between soil P:N and (a) plant aboveground and (b) belowground C:N ratios. Also relationships between soil N:P and (c) plant aboveground, and (d) belowground C:P ratios. Gray shaded areas indicate 95% confidence interval limits. NS, nonsignificant.

Effects of grazing, nutrient, and liming applications on plant stoichiometry

Grazing by rabbits contributed to decreased plant aboveground and belowground C:N ratios as well as plant aboveground C:P ratios. These findings partly confirm our first hypothesis of grazing-induced increases in plant N and P tissue concentrations. Previous studies suggest that the increased concentration of nutrients in plant tissues is mediated by positive grazing-induced effects on soil N cycling, which leads to greater plant N uptake (Shan et al. 2011, Bai et al. 2012, Zheng et al. 2012, Krumins et al. 2015). Grazers are responsible for returning significant levels of available forms of N to the soil (McNaughton et al. 1997), thus influencing the “relative” allocation of C and N in plant tissues (Holland et al.

1996, Bardgett et al. 1998, Olff and Ritchie 1998, Zheng et al. 2012). In our study, we observed higher rates of soil N mineralization in grazed grasslands, which agrees with previous findings (Coetsee et al. 2010) suggesting that grazers can significantly influence plant C:N stoichiometry. We did not observe, however, any change in plant N:P ratios under grazing and this could be because grazers also contributed to increase available forms of P in our grassland soils.

Contrary to our second hypothesis, we found that liming had a positive effect on plant aboveground C:N ratios and a negative effect on plant belowground N:P and C:P ratios. Increases in plant C:N ratios may be related to increases in plant aboveground productivity in limed plots possibly because of liming-induced increases in

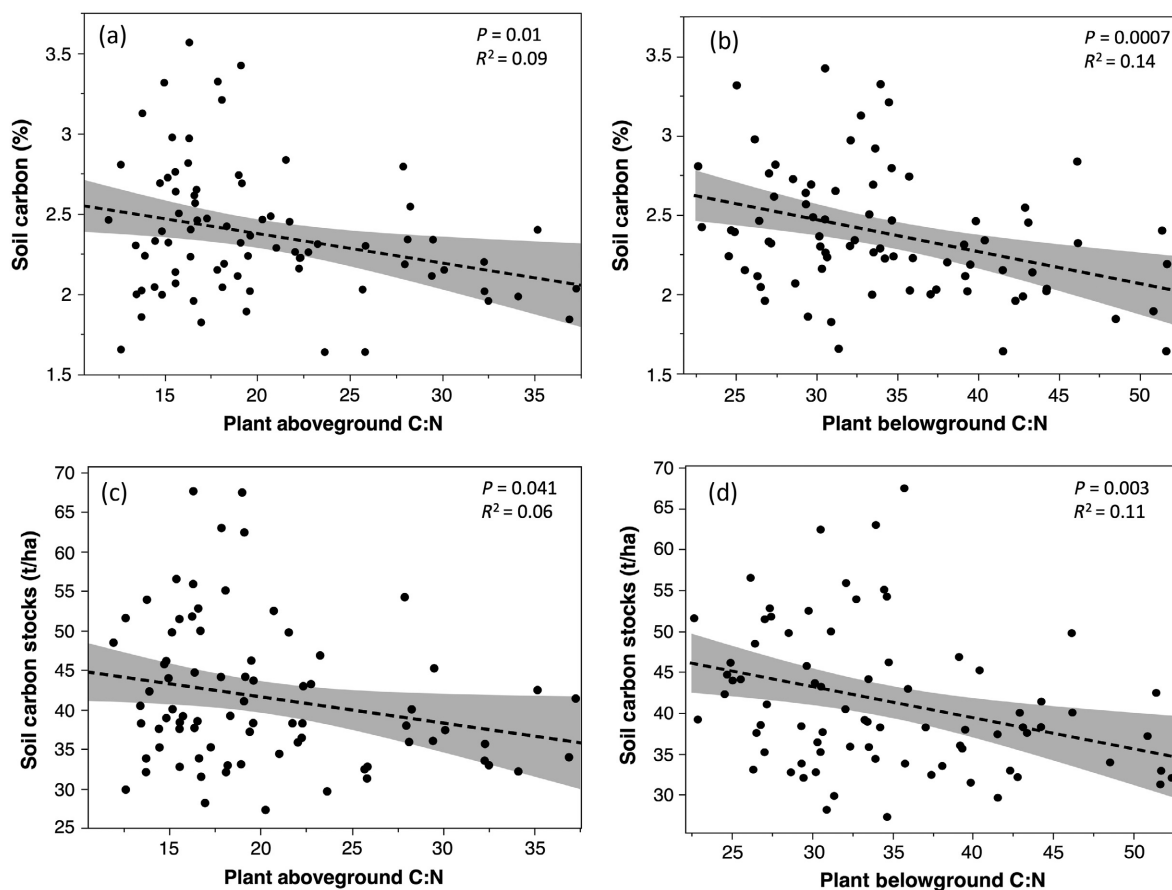


Fig. 6. Relationships between soil C content (%) and (a) plant aboveground and (b) belowground C:N ratios. Also relationships between soil C stocks (t/ha) and (c) plant aboveground and (d) belowground C:N ratios. Gray shaded areas indicate 95% confidence interval limits.

soil cation exchange capacity and soil nutrient availability (Johnson et al. 2005, Fornara et al. 2011; Holland et al. 2018). Decreases in root N:P and C:P ratios may suggest that liming has a positive effect on root P concentrations. It has been suggested that liming decreases the availability of P in soils due to the binding of phosphate with the calcium (Ca) component of agricultural lime (Aerts et al. 2003, Olsson et al. 2009, Saarsalmi et al. 2011). It could be, however, that liming has a positive effect on arbuscular mycorrhizal fungi (AMF) colonization (Johnson et al. 2005, Guo et al. 2012), which can greatly improve plant P uptake explaining why plant roots from limed soils have higher P content.

We found that the chronic addition of inorganic nutrient fertilizers to soils led to predictable plant stoichiometric responses. For

example, confirming our third hypothesis, we found that plant above- and belowground C:N ratios significantly decreased under long-term N addition (i.e., C:N ratios were highest in control plots and P-only fertilized plots). We also found that plant C:P ratios were highest in control and N-only fertilized plots but lowest in plots receiving inorganic P additions (either P alone or in combination with other nutrients). According to our third hypothesis, we found that plant aboveground and belowground C:N ratios were negatively related to net soil N mineralization rates (Fig. 4a, b). Soil Olsen P values (an index of P availability in soils) were significantly negatively related to both plant aboveground and belowground C:P ratios (Fig. 4c, d), also suggesting that when soil P availability decreases, plant C:P ratios increase.

Moreover, our evidence is that the long-term addition of only one of the two nutrients (either N- or P-only) will cause a significant reduction of the other element content in plant tissues relative to C (i.e., high plant C:P under long-term N-only additions or high C:N under long-term P-only additions; Fig. 3a, b). It is not clear how these stoichiometric responses might relate to changes in N:P co-limitation. Assuming that plant N:P ratios <10 would indicate N limitation (Güsewell 2004, Craine et al. 2008), we found that 89% of our experimental plant communities could be considered N-limited except for the N-only fertilized grasslands. We found also evidence, however, that plant C:P ratios increased with increases in soil N:P ratios (Fig. 5c, d), suggesting that P concentration in plant tissues is positively related to the relative availability of P compared to N in soils. This was not, however, the case for plant C:N ratios, which did not increase at increasing soil P:N ratios (Fig. 5a, b), a finding which could have different explanations. For example, this could depend on our limited ability to simultaneously measure N and P availability in soils for plant uptake under field conditions and across temporal and spatial scales. Also plant-microbial interactions (i.e., symbioses, such as AMF) may alter rates of N and P uptake by plants regardless of the relative availability of N and P in soils. Finally, the presence and role of particular functional groups (i.e., legumes) able to fix atmospheric N may contribute to provide N to other functional groups, thus uncoupling plant C:N ratios from actual N availability in soils (Di Palo and Fornara 2015).

Plant C:N:P ratios and soil element cycling and storage

We found that net soil N mineralization rates were negatively related to both aboveground and belowground C:N ratios. Our prediction was that as plant C:N ratios decreased under chronic N additions, soil N availability and cycling should increase because of positive N-induced effects on soil N mineralization rates (Mooshammer et al. 2014). This was confirmed for both aboveground and belowground plant compartments. Similarly, we found plant C:P ratios decreased as soil available P increased, and there was a strong negative relationship between plant C:P ratios and soil Olsen P values.

Our final hypothesis was that nutrient-rich soil environments would be associated with lower plant C:N and C:P ratios (which we found in our study), and with reduced soil C pools when compared to nutrient-poor soil environments (Hobbie 1992, Sterner and Elser 2002, Hessen et al. 2004, Wardle et al. 2004). We expected grassland soils receiving long-term applications of N-fertilizer show smaller soil C content and soil C stocks when compared to soils receiving no nutrients. Our results show quite the opposite trend, whereby soil C stocks increased as plant C:N ratios decreased in chronically fertilized grasslands (Fig. 6). It is very unlikely that greater plant aboveground productivity in fertilized grassland plots would positively contribute to soil C accrual through higher C returns to soils, mainly because most biomass is annually removed by either grazing or mowing. It could be that N-fertilized soils, which support plant communities with low C:N, ratios are associated with greater soil C sequestration partly because of positive nutrient-induced effects on soil microbial activities (Cotrufo et al. 2013, Cenini et al. 2015, Zechmeister-Boltenstern et al. 2015). For example, Cenini et al. (2015) show how an increase in microbial energy demand (i.e., C demand) as a result of low C:N plant detritus inputs in the same fertilized grasslands will increase the activity of the C-acquiring enzyme β -1,4-glucosidase. The increased activity of this enzyme was then linked to more C being processed and accumulated (including microbial biomass C) into organo-mineral soil pools. It could be that when soil microbial communities become C-limited (as in our chronically fertilized grasslands), microbial metabolism switches from N to C resource supply, and thus an increase in C-use efficiency was determined, which is associated with high microbial C sequestration potential (Zechmeister-Boltenstern et al. 2015). These studies suggest that the return of low C:N plant detritus to soils can contribute to increasing microbial C-use efficiency, which then determines more microbial C being accumulated and stabilized into soil organo-mineral fractions, thus contributing to greater soil C stocks. It is also true, however, that soil C stocks in our fertilized grassland were particularly higher under the N-only treatment and lower when N was added with other nutrients (e.g., P, K, Mg). It is possible that greater soil C stocks under the N-only treatment are

partly related to increases in total root mass (Fornara et al. 2013) and thus to increases in C inputs to soils. Further research could address how changes in soil N availability interact with changes in the availability of other key nutrients to affect plant–soil–microbe interactions and ultimately the ability of managed grassland soils to act as atmospheric CO₂ sinks.

CONCLUSION

Our study is one of the first to show how multiple long-term grassland management practices (i.e., grazing, liming and nutrient fertilization) can influence plant C:N:P stoichiometry. Plant element ratios respond to changes in soil nutrient availability in ways, which are predicted by ecological stoichiometry theory. However, we found that increases in plant C:N ratios do not predict long-term increases in soil C content and C pools. On the contrary, nutrient-fertilized grassland soils dominated by plant communities with relatively low C:N ratios have the potential to sequester more C than unfertilized grassland communities characterized by higher plant C:N ratios. The underlying biogeochemical mechanisms responsible for these effects could have far-reaching implications for the management of grassland ecosystems, especially in relation to long-term changes in soil C sequestration.

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