
Fornara, Dario A.; **Banin, Lindsay**; Crawley, Michael J.. 2013 Multi-nutrient vs. nitrogen-only effects on carbon sequestration in grassland soils. *Global Change Biology*, 19 (12). 3848-3857. 10.1111/gcb.12323

Multi-nutrient vs. nitrogen-only effects on carbon sequestration in grassland soils

Running head: Nutrient addition and soil C storage

Authors: Dario A. Fornara[1*], Lindsay Banin[1,2], Michael J. Crawley[3]

[1] Environmental Sciences Research Institute, University of Ulster, Coleraine BT52 1SA, UK; [2]Centre for Ecology and Hydrology, Bush Estate, Penicuik, Midlothian, EH26 0QB; [3]Imperial College, Silwood Park, Ascot, Berkshire SL5 7PY, UK

D. A. Fornara, email: d.fornara@ulster.ac.uk

L. Banin, email: lindsay.banin@gmail.com

M. Crawley, email: m.crawley@imperial.ac.uk

Corresponding author:

[*]Dario A. Fornara

Environmental Sciences Research Institute, University of Ulster, Coleraine,

BT52 1SA, UK

Email: d.fornara@ulster.ac.uk; Phone: +44 (0) 28 70324658; Fax: +44 (0) 2870 324911

Key words: ecosystem services, grasslands, nitrogen fertilization, nitrogen losses, nutrient addition, plant productivity, root mass

Type of paper: Primary Research Article

Abstract

Human activities have greatly increased the availability of biologically active forms of nutrients (e.g. nitrogen (N), phosphorous (P), potassium (K), magnesium (Mg)) in many soil ecosystems worldwide. Multi-nutrient fertilization strongly increases plant productivity but may also alter the storage of carbon (C) in soil, which represents the largest terrestrial pool of organic C. Despite this issue is important from a global change perspective, key questions remain on how the single addition of N or the combination of N with other nutrients might affect C sequestration in human-managed soils. Here, we use a 19-year old nutrient addition experiment on a permanent grassland to test for nutrient-induced effects on soil C sequestration. We show that combined NPKMg additions to permanent grassland have 'constrained' soil C sequestration to levels similar to unfertilized plots whereas the single addition of N significantly enhanced soil C stocks (N-only fertilized soils store, on average, 11 t C ha^{-1} more than unfertilized soils). These results were consistent across grazing and liming treatments suggesting that whilst multi-nutrient additions increase plant productivity, soil C sequestration is increased by N-only additions. The positive N-only effect on soil C content was not related to changes in plant species diversity or to the functional composition of the plant community. N-only fertilized grasslands show, however, increases in total root mass and the accumulation of organic matter detritus in top-soils. Finally, soils receiving any N addition (N-only or N in combination with other nutrients) were associated with high N losses. Overall, our results demonstrate that nutrient fertilization remains an important global change driver of ecosystem functioning, which can strongly affect the long-term sustainability of grassland soil ecosystems (e.g. soils ability to deliver multiple ecosystem services).

Introduction

Since the formulation and dissemination of the "Law of the Minimum" in agricultural chemistry (Sprengel, 1828; Liebig, 1840) several studies over the last two centuries have acknowledged the importance of synergistic interactions between multiple nutrients for increasing plant yields across a broad range of human-managed ecosystems (Viets, 1977; Berck & Helfand, 1990; Elser *et al*., 2007; Harpole *et al.*, 2011). The addition of multi-nutrients to soils is a very common practice worldwide mainly because chemical fertilization greatly increases plant productivity, which is an important ecosystem service to human societies (MA, 2005). However, adding biologically available forms of nutrients to soils may lead to perturbations in the cycle and storage of C in belowground compartments. Soils form the largest active terrestrial pool of organic C and changes in their ability to act as terrestrial C sinks may have global change implications (Post *et al*., 1982; Smith *et al.* 1997).

Previous studies have focused on the potential role of one nutrient, N, in affecting soil C cycling and storage across very different ecosystems (de Vries *et al.,* 2006; Pregitzer *et al.,* 2008; Janssens *et al.,* 2010; Liu & Greaver, 2010; Lu *et al.,* 2011; Fornara & Tilman, 2012). These studies have yielded mixed results and show that N additions may have either positive, negative or no effect on total soil C pools. Such variable responses could have multiple environmental causes but could also suggest that net N effects on soil C cycling and storage might depend on the addition or on the presence/absence of other nutrients in soils (Townsend *et al.,* 2011). For example the addition of P and K to soils can differently affect rates of plant litter decomposition (Kaspari *et al*., 2008) thus potentially influencing soil C accumulation. Key questions remain about which nutrients other than N or what combinations of nutrients (e.g. potential synergistic nutrient effects) might ultimately affect soil C sequestration. Answering these questions is crucial if we want to make more accurate predictions on long-term multi-nutrient effects on soil C sequestration.

Potential long-term nutrient effects (other than N) on soil C sequestration have rarely been addressed. Mack *et al.* (2004) show that NP fertilization contributed to decreases in soil organic C pools in tundra ecosystems. The evidence from two experiments on tropical forest soils suggests that P additions were responsible for increases in soil CO_2 respiration thus causing soil C losses (Cleveland & Townsend, 2006; Cleveland *et al.*, 2006). In contrast with these findings there is also evidence that P-only additions could lead to greater C sequestration in soils (Bradford *et al.*, 2008). Not surprisingly, most of the information on multi-nutrient effects on soil C sequestration result from agricultural studies in intensively managed cropping systems. These studies show for example that soil C sequestration under NPK applications is significantly lower than C sequestration under organic manure/compost applications (Böhme *et al.*, 2005; Markgraf *et al.*, 2012; Moharana *et al.*, 2012; Yu *et al.*, 2012).

Poor mechanistic understanding of single (e.g. P, K) or multi-nutrient (e.g. NPK) effects on soil C sequestration also hampers our ability to make predictions on the long-term sustainability of soil ecosystems (e.g. their ability to deliver multiple ecosystem services). Among human-dominated ecosystems, grasslands in agricultural use retain high economic value and cover a significant proportion of landmass across central and Western Europe (Eurostat, 2011). For example, in the UK alone there are 5.1 million hectares of improved grasslands (Carey *et al.*, 2008), many of which have historically received multi-nutrient additions. Soil organic matter formation in these grasslands is regulated by the availability of multiple nutrients, of which positive synergistic effects on above-ground grass yields are well known, but whether and how these effects might also alter soil C cycling is highly uncertain.

Here we specifically ask how the long-term addition (i.e. 19 years) of different nutrient treatments (i.e. N, P, K in isolation and NP, PK and NPKMg in combination) has affected soil C sequestration in a mesotrophic permanent grassland in Berkshire, UK. Previous studies in this grassland experiment have addressed potential herbivore-nutrient interactions on plant community structure, composition and plant aboveground productivity (Edwards *et al.,* 2000; del-Val & Crawley, 2005; Allan & Crawley, 2011). In this study we primarily ask whether and how different chronic fertilization treatments have influenced soil C sequestration when compared to unfertilized grasslands. Because chronic N fertilization may reduce organic matter decomposition in top-soils (Pregitzer *et al.,* 2008; Janssens *et al.,* 2010) whereas long-term NP fertilization may decrease soil organic C pools (Mack *et al.,* 2004), our hypothesis, is that long-term additions of non-N nutrients (e.g. P or K) to soils in combination with N will determine lower soil C sequestration when compared to N-only additions. We also consider changes to above and belowground plant biomass, plant species diversity and plant functional group composition, in association with nutrient additions, to develop a better mechanistic understanding of any observed changes to soil C stocks.

Methods

Study site

We conducted our study in Nash's Field, a mesotrophic grassland (MG5*, Lolio-Cynosuretum cristati* grassland, *Anthoxanthum odoratum* sub-community; Rodwell, 1992) lying on acid, sandy soils of the Bagshot series in Silwood Park, Berkshire. Historically, Nash's Field was managed as a hay meadow from 1947 to 1990, with a single mid-summer cut in most years, and a second, autumn cut, taken in years of unusually high grass production (Crawley, 1990). The soils of Nash's Field are characterised by low P status, for example measurements made in 1991 indicated that P values were very low (5.6±0.47 mg kg^{-1} NaHCO3 soluble P). Levels of exchangeable K were relatively high (88.4±7 mg kg^{-1}) as were levels of Mg (37.5±21 mg kg^{-1}) thus unlikely to be limiting to grass growth. Modelled total atmospheric N deposition in this area of the UK (Harpenden, Hertfordshire) has been estimated as be ~22 kg N ha^{-1} y^{-1} (http://www.apis.ac.uk), which we assumed to be even across our Nash's Field experiment. To prevent invasion by woody plants, the fenced plots were managed as a hay meadow, with a single hay cut in early August each year, using a sickle bar mower. The experiment was established in the summer of 1991 and was designed to test hypotheses about the interaction of herbivory and nutrient additions in determining plant community structure. In this paper we concentrate on the effects of nutrients addition, lime addition, and herbivore exclusion on soil C sequestration. The experiment is a 4-factor factorial, laid out in a split-plot design (see Supplementary Fig. 1). The four largest plots are used for invertebrate herbivore exclusion (e.g. plus and minus insects), then each of these plots is split in half for the exclusion of larger vertebrate herbivores (plus and minus rabbit fences), and each of these, in turn, is split into two soil pH treatments (limed and unlimed). Lime was applied each January at a rate of 5 tonnes of $CaCO_3$ ha^{-1}.

Within the liming treatments 7 plots (2m×2m) were laid out for the application of various combinations of mineral nutrients (N, P, K and Mg). Specifically, four fertiliser nutrients (N as ammonium nitrate, K as muriate of potash, P as triple superphosphate, and Mg as Epsom salts) have been applied at the following rates: N at 100 kg ha^{-1}, P at 35 kg ha^{-1}, K at 225 kg ha^{-1} and Mg at 11 kg ha^{-1} during the first week in April, each year from 1992 to 2011. We sampled experimental plots that received the following fertiliser treatments: (1) N-only, (2) P-only, (3) K-only, (4) N and P together, (5) P and K together, (6) simultaneous addition of all nutrients (N, P, K and Mg), and (7) no addition (control treatment). Thus 4 large plots × 2 grazing treatments (± rabbit herbivory) × 2 liming applications (± lime) × 7 nutrient treatments = 112 plots (each 2×2 m).

Soil sampling and analyses

Soil samples for the analysis of C, N and P, were collected in July 2011 to a soil depth of 20 cm (using a 3 cm diameter soil corer) at four locations within each of the 112 experimental plots. Before collecting the soil, plant litter, if present, was removed. Soils were then sieved to 2 mm to remove roots, dried at 40 °C for 5 days, ground and analysed for total C and N by combustion and gas chromatography (COSTECH Analytical ECS 4010 instrument). Ash samples analysed after furnace burning (16 hours at 550 °C) revealed negligible inorganic C. Soil sub-samples collected in each plot were air-dried and ball milled to pass a 150 µm sieve and then processed for the determination of total soil P by sodium hydroxide fusion (Smith & Bain, 1982). Two extra soil samples per plot were collected to 20 cm soil depth using a PVC corer 8 cm in diameter to measure soil bulk density (i.e. mass of dried soil divided by soil volume).

Potential net soil N mineralization rates

We performed laboratory incubations of soils collected from each of the 112 plots in May 2011 to assess net N mineralization rates. Soil samples were collected to 20 cm soil depth from three sites within each plot, extracted with 1M KCl, shaken for 0.5 h, settled overnight at 4° C and analyzed for NH_4^+ -N and NO_3^- -N with a Bran-Luebbe AA3 auto analyzer. An additional 25-g subsample from each plot was incubated for 30 days in a dark room at 22° C after roots were sieved out (see Fornara & Tilman, 2008). Sufficient water was added to each sample to keep moisture constant. After 30 days soil samples were extracted and analysed for NH_4^+ -N and NO_3^- -N as above. To determine net N mineralization, final extractable concentrations of NH_4^+ and NO_3^- per gram of soil were subtracted from initial extractable concentrations per gram of soil.

Plant belowground sampling and analyses

In May 2011 the 112 plots were sampled for plant belowground mass by collecting four evenly spaced soil cores in each plot (each core was 5 cm in diameter and collected to 20 cm depth). Samples were washed gently with water over a fine mesh screen until roots were free of soil. Roots were then dried; any soil residual was removed and roots were then weighed. Belowground biomass was dried at 65°C to constant mass and then ground and analysed for total C and N following standard methods on a 1500 NA Carlo-Erba element analyzer (Elan Tech., NJ). Total P in root material was analysed following 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 (see also Murphy & Riley, 1962).

Plant aboveground biomass and plant percent cover

To address potential changes in both plant aboveground biomass and plant community composition across experimental treatments, which may help explain changes in soil C, we analyzed two sub-sets of available data from this long-term experiment. First, plant aboveground biomass was measured in 1997 in each of our 112 experimental plots. In August 1997 a single sampling quadrat measuring 50cm x 25cm (selected at random from the central 1m x 1m section of each 2m x 2m plot to avoid edge effects) was cut to ground level using scissors. Fresh samples were returned to the laboratory separated at the plant-species level, dried at 80 °C for 24 hours and weighed. Second, in June 2012 the number of plant species as well as the percentage cover of each plant species in each experimental plot was estimated (percentage cover was agreed by two investigators for each species, plus any bare ground). Plants were grouped into three functional types: C3-grasses, legumes and forbs (see Fornara & Tilman, 2008).

Data analysis

We used a mixed effects ANOVA in which the four large plots (block) in the split-plot design were treated as random effects and the other treatment factors (insects, rabbit grazing, liming and nutrient addition treatments) were all fixed effects. We tested these treatments effects on different ecosystem response variables (i.e. soil C, soil N, root mass, root stoichiometry, plant species richness, plant aboveground biomass, plant cover). Since the insects, grazing and liming treatments did not account significantly for variation in the response variables, simpler models were produced which included only ‘nutrients’ as fixed effects (see example output in Supplementary Table 1). Final models were produced using restricted maximum likelihood (REML) method. We checked that final models conformed to modeling assumptions. Significant differences between factor levels (i.e. the seven nutrient treatments) were tested using post-hoc Tukey test in the package multcomp. All analyses were conducted using the lme4 package in R version 2.10.1 (R Development Core Team 2008).

Linear regression analyses were used to explore potential covariation between soil C content and other belowground variables. We also calculated the difference in total soil C (and N) pools between each nutrient-fertilized plot and the average of the controls, and then divided this value by the cumulative amount of N added to that plot over 19 years. Thus we estimated the units of soil C gained per units of N added (i.e. “C gain efficiency” expressed as: g C/g N added) and the units of soil N retained per units of N added (i.e. “N retention efficiency” expressed as: g N/g N added). We explicitly refer to ‘N retention’ and ‘C gain’ because our grasslands did receive experimental N inputs but not C inputs.

Results

N-only addition and soil C pools

We found that total soil C pools were significantly affected by nutrient additions but not by other experimental treatments such as the presence of insects, rabbit grazing or liming. In particular, total soil C pool was higher under the N-only fertilization treatment (Fig 1; Supplementary Table 1) and significantly higher for the N-only treatment than all other treatments except PK ($P < 0.05$, Tukey *post-hoc* test). Net soil C change between the N-only treatment and the control was 3 times greater than the net soil C change between any of the other fertilization treatments and the control. Similarly total soil N pools and net soil N changes were both significantly higher under the N-only fertilization treatment (Supplementary Fig. 2). The positive N-only effect on total soil C pools occurred regardless of the application of lime (Fig. 2a). There is, however, increased C accrual in limed soils under the P-only and PK nutrient treatment (Fig. 2a). Positive N-only effects on total soil C pools also occurred regardless the presence/absence of rabbit grazing (Fig. 2b), although there were increases in soil C in grazed plots receiving no nutrients (control) and K-only and PK nutrient additions.

N-induced effects on soil C pool 'constrained' by multiple nutrient additions

We found a significant interaction between N and P ($P = 0.01$) on soil C stocks (i.e. soil C was significantly lower when NP where added together than when N was added alone). Further analyses also show that the simultaneous addition of key nutrients, such as P and K together with N, contributed to reduce soil 'C gain efficiency' (Fig. 3a, b) and soil 'N retention efficiency' (Fig. 3c, d) per unit of N added when compared to N-only addition. This means that N-only fertilized soils gained more C (and retained more N) per unit of N added when compared to soils that received N in combination with P or K. The positive effect of N-only fertilization on soil 'C gain efficiency' occurred across a wide range of soil pH varying from pH<5 to pH>7 (Supplementary Fig. 3a). Thus, when N, P, K (and Mg) are simultaneously added to soils, the ability of these soils to store 'extra' C per unit of N added tended to decrease regardless of changes in soil pH. Soil pH was strongly affected by liming but not by grazing (Supplementary Fig. 3b, c).

Nutrient effects on plant aboveground biomass and functional group composition

Plant aboveground biomass, as measured in 1997 in the same experimental plots and six years after the start of the experiment, was strongly increased by the addition of multiple nutrients simultaneously (Fig. 4a). We did not find any significant effect of nutrient treatments on the aboveground biomass (as measured in 1997) of different plant functional groups. Regardless of nutrient fertilization treatment C3 grasses represented at least 73% of the total aboveground biomass in these experimental plots (Fig. 4b). Biomass of non-leguminous forbs varied between 10% and 16% whereas legumes represented <0.1% of plant community biomass. However, in 2012 we found that the three nutrient treatments including N (i.e. N-only, NP and NPKMg) were associated with the lowest number of plant species (Fig. 5a) and the highest proportion cover of C3 grasses (C3 grasses cover ~70% of plot area) when compared to control, K-only, or PK nutrient additions (Fig. 5b).

Nutrient effects on plant belowground compartments and soil & root stoichiometry

There was no significant difference in total root mass across the nutrient treatments although the addition of multiple nutrients tended to decrease root mass whereas N-only additions increased root mass: the addition of N alone yielded mean root mass 25.8% higher than the control, 34.5% greater than P-only and 44.4% greater than plots with all nutrients added (Fig. 6). Similarly, we did not find any significant nutrient effect on total root C and N pools (Supplementary Fig. 4).

Grassland soils receiving N additions have retained very little of this N input by 2011 (Fig. 7a). In particular NPKMg-treated soils have lost 98% of the total N added over 19 years (1.86 t N ha^{-1} loss *vs*. 1.9 t N ha^{-1} added). The same NPKMg-treated soils have lost 29% of total P added over 19 years (0.2 t P ha^{-1} loss *vs*. 0.665 t P ha^{-1} added; Fig. 7b). NP-treated soils lost 85% of N added and 16% of P added over 19 years, thus in the long-term altering soil NP stoichiometry (Supplementary Fig. 5). Finally, N-only treated soils lost 64% of N added during the same period of time (Fig 7a). The availability of key nutrients in soils such as P and N was strongly influenced by nutrient treatment. For example, Olsen-P varied between 102 and 113 (mg P/l soil) in soils where P was added (All, P, NP treatments) but was < 4 (mg P/l soil) in control plots and N-only fertilized soils. Net soil N mineralization was highest in NP-fertilized plots (~ 18 g N/kg soil) and lowest in P-only fertilized plots (~ 9 g N/kg soil). We found that nutrient treatments have significantly affected soil C:N ratios; for example, soil C:N was significantly lower in N-only treated soils than those treated with P, K or PK (Tukey *post-hoc* test, $p < 0.05$; Supplementary Fig. 5a), soil C:P ratios (Supplementary Fig. 5a) and soil N:P ratios (Supplementary Fig. 5a). Similarly, we found that nutrient treatments had significant effects on root C:N ratios; root C:N was significantly lower in samples receiving N, NP and NPKMg than the treatments excluding N (control, K, P and PK) (Supplementary Fig. 5b). Root C:P ratios were significantly lower in all treatments including the application of P (Supplementary Fig. 5b) and root N:P ratios also varied significantly across treatments (Supplementary Fig. 5b). The only significant relationship between any root stoichiometry ratio and total soil C pools was a negative correlation between root C:N and soil C pool ($F_{1,110} = 14.9$, $P = 0.0002$; Supplementary Fig. 6).

Discussion

The ‘N-only’ effect on soil C sequestration

Our study shows that 19 years of N-only additions (in the form of ammonium nitrate) strongly increased soil C storage in these mesotrophic grasslands. A potential mechanism underlying this C accrual could be the long-term accumulation of partially undecomposed plant-derived detritus that we often observed in topsoil layers of the N-only fertilized plots. This observation is supported by previous findings, which show that chronic N additions can greatly reduce microbial mineralization of soil organic pools, effectively slowing down decomposition (Janssens *et al.*, 2010; Liu & Greaver, 2010), and thus promoting organic C accumulation in these soils. Similar findings (Ramirez *et al.*, 2012) show that, across very different terrestrial ecosystems, reduced decomposition was associated with negative N-induced effects on the activity of different soil extra-cellular enzymes including those responsible for cellulose breakdown. Our results clearly show that both soil ‘C gain efficiency’ and soil ‘N retention efficiency’ (Fig. 3) were significantly greater in N-only fertilized plots and did not change across a broad range of soil pH values (Supplementary Fig. 3a). This suggests a higher rate of soil C and N accumulation in these plots that could be partially explained by reduced decomposition of soil organic pools.

Lack of a multi-nutrient effect on soil C sequestration

The addition of P and K either in isolation or in combination with N (i.e. NP and NPKMg) and without N (i.e. PK) was associated with low soil C sequestration (Fig. 1). The lack of a multi-nutrient effect on soil C content agrees with previous findings, which show that NPK additions can ‘constrain’ soil C sequestration in intensive croplands

(Tong *et al.,* 2009; Yu *et al.,* 2012). Here we show that multi-nutrient additions may also reduce soil C sequestration in permanent grasslands. This finding, if proved general, could have far reaching implications for the ability of these grasslands (1) to act as soil C sinks under current global change, and (2) to deliver multiple ecosystem services to human society. In the UK there are 5.1 million hectares of improved grasslands (Carey *et al.,* 2008) and although NPK fertilization rates (and organic manure applications) may greatly vary depending on grassland management (Defra, 2010), the question remains whether agriculturally improved grasslands may function as active soil C sinks or not. Previous studies show that soil C stocks of many grasslands across England and Wales may not change (or their soil C stocks may even decrease) over decades (Bellamy *et al.,* 2005; Smith *et al.,* 2007). Also, a recent study using soil data spanning 129 years of the Park Grass Experiment at Rothamsted, UK, shows that the organic C content of fertilized (and unlimed) soils did not change significantly between 1876 and 2005 (Fornara *et al.,* 2011). Finally, in our study we found that the lack of positive multi-nutrient (NPKMg) effects on soil C pools was consistent (Fig. 2a, b) across liming and grazing treatments, two very common agricultural practices. Liming and grazing treatments, however, tended to have positive effects on soil C sequestration under specific nutrient treatments. For example, liming increased soil C content under the PK treatment (Fig. 2a) and this was possibly due to positive liming- and PK-induced effects on the abundance of N-fixing legume species (unpublished data) which are known to have positive effects on soil C accumulation (Fornara & Tilman, 2008). Grazing contributed to enhance soil C content of control plots (and K-only treated plots) and this was possibly due to grazing-induced increases in soil N availability as we found greater soil N mineralization in grazed plots (Supplementary Fig. 7).

Nutrient additions and changes in above- and belowground plant mass

In our mesotrophic grassland, multi-nutrient additions had strong positive effects on plant aboveground biomass production (Fig. 4a). Our evidence is that by 2012 the chronic addition of N to soils either alone or in combination with other nutrients (i.e. N-only, NP and NPKMg) was responsible for (a) a strong decrease in plant species diversity and (b) an increase in C3-grass cover in our experimental plots (Fig. 5a, b). However, neither decreases in plant species diversity nor increases in C3 grass cover were significantly related to changes in soil C stocks across our experimental plots.

On the other hand we found that total root mass was greatest under the N-only treatment and lowest under the addition of all nutrients: these differences were not significant, owing to the high variability among plots under the same treatment (Fig. 6). However, because roots substantially contribute to total soil C inputs (Trumbore & Gaudinski 2003; Farrar *et al.,* 2003), these differences in total root mass between N-only fertilized plots and all other experimental plots might partly explain changes in the C sequestration ability of these soils. A recent review, which included 257 published studies, shows that N-induced changes in soil C storage can be positively related to changes in root biomass (Lu *et al.*, 2010). Similar findings from a long-term grassland experiment in North America show that N-induced increases in root mass were associated with greater soil C sequestration (Fornara & Tilman, 2012).

Effects of nutrient treatments on plant aboveground mass were distinct from their effects on plant belowground mass (see Figs 4a and 6) and to a degree, were opposite. This may indicate differences in biomass allocation dependent on nutrient availability. We suggest that nutrient-induced effects on both root mass accumulation and root decomposition may partly explain changes in soil C sequestration. Further studies could address, for example, whether N-induced increases in root mass are due to increases in root productivity or to the accumulation of un-decomposed root material across years. On the other side reduced root mass in NPKMg-fertilized plots may reflect preferential allocation of plants to aboveground biomass when all nutrients are available and it provides little competitive advantage to produce comparatively larger root systems.

Finally, it might be that NPKMg additions to agricultural grassland soils do not influence C sequestration because most of plant aboveground biomass is removed for forage production thus reducing any positive NPKMg effect on C inputs to soil from plant aboveground compartments. Plant biomass removal and also potential N losses (via leaching and gaseous emissions) from NPKMg-fertilized soils may also explain why only 2% of the total N added over 19 years actually remained in these soils by 2011 (Fig. 7a).

Lack of multi-nutrient effects on soil C sequestration

Multiple studies from both cropland and grassland systems show that long-term NPK fertilization determines a loss of soil structure (Ciarkowska, 2010; Herencia *et al.*, 2011; Markgraf *et al.*, 2012). In particular the proportion of water stable soil macro-aggregates (e.g. > 250 µm) tends to decrease (or not significantly change) under NPK additions when compared to organic manure applications (Aoyama *et al.*, 1999; Bandyopadhyay *et al.,* 2010). The loss of stable soil macro-aggregates can negatively affect soil C sequestration because macro-aggregates represent a large proportion of total soil mass (Six *et al.*, 2000). Macro-aggregates structure includes various binding agents such as microbial- and plant-derived polysaccharides, which are all C-rich materials (Tisdall & Oades, 1982). Thus reduced soil structure under chronic NPK fertilization would mean a decrease of C-rich macro-aggregates and thus a loss of soil organic matter.

Previous studies across very different grassland systems also show (1) a decrease in microbial C biomass (Marschner *et al.*, 2003; Moharana *et al.*, 2012), (2) a decrease in microbial functional diversity (Islam *et al.*, 2011), and (3) an increase in bacterial-dominated (versus fungal-dominated) microbial communities under multi-nutrient inorganic fertilization (Smith *et al.*, 2008; de Vries & Bardgett 2012). However, identifying causal relationships between changes in microbial community functions and changes in soil organic C sequestration remains difficult, partly because soil microbial activities may vary even within similar multi-nutrient additions (Böhme *et al.*, 2005), and partly because of the difficulty in linking microbial processes to long-term changes in soil C stocks.

Overall our study shows that N-only additions to permanent grassland are responsible for significant increases in soil C sequestration whereas multi-nutrient fertilization is associated with unenhanced soil C sequestration. Thus the positive multi-nutrient effect on plant productivity may not apply to soil C sequestration raising questions about the ability of these grasslands to deliver multiple ecosystem services (i.e. high plant yields alongside high soil C sequestration and low N and P leaching). Nutrient addition to soils remains an important global change driver of ecosystem structure and functioning and its potential effects on the cycle and storage of C in soils need to be further addressed by interdisciplinary research. Further studies could address how varying organic and inorganic nutrient additions to soils might affect (1) C and N sequestration, (2) plant above- and below-ground mass allocation, (3) microbial activities related to the storage and cycling of different nutrients, and (4) critical soil physical properties in order to improve the delivery of multiple ecosystem services by human-managed grasslands.

Acknowledgements

We thank Eleonora Fitos for her assistance during fieldwork. Thanks to Valeria Cenini, Francesca Di Palo, Rachael Ramsey and Cathleen McFadden for their assistance in laboratory analysis of samples. We are also grateful to the subject matter Editor and three anonymous reviewers for their helpful comments on previous versions of this paper.

Literature

Allan E, Crawley MJ (2011) Contrasting effects of insect and molluscan herbivores on plant diversity in a long-term field experiment. *Ecology Letters*, **14**, 1246-1253.

Aoyama M, Angers DA, N'Dayegamiye A (1999) Particulate and mineral-associated organic matter in water-stable aggregates as affected by mineral fertilizer and manure applications. *Canadian Journal of Soil Science,* **79**, 295-302.

Bandyopadhyay KK, Misra AK, Ghosh PK, Hati KM (2010) Effect of integrated use of farmyard manure and chemical fertilizers on soil physical properties and productivity of soybean. *Soil & Tillage Research,* **110**, 115–125.

Bellamy PH, Loveland PJ, Bradley RI, Lark RM, Kirk GJD (2005) Carbon losses from all soils across England and Wales 1978–2003. *Nature*, **437**, 245–248.

Berck P, Helfand G (1990) Reconciling the von Liebig and Differentiable Crop Production Functions. *American Journal of Agricultural Economics*, **72**, 985-996.

Böhme L, Langer U, Böhme F (2005) Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. *Agriculture, Ecosystems and Environment,* **109**,141–152.

Bradford MA, Fierer N, Jackson RB, Maddox TR, Reynolds JF (2008) Nonlinear root-derived carbon sequestration across a gradient of nitrogen and phosphorous deposition in experimental mesocosms. *Global Change Biology,* **14**, 1113-1124.

Carey PD, Wallis S, Chamberlain PM *et al.* (2008) *Countryside survey: UK results from 2007*. NERC/Centre for Ecology & Hydrology, 105 pp.

Cleveland CC, Townsend AR (2006) Nutrient additions to a tropical rain forest drive substantial soil carbon dioxide losses to the atmosphere. *Proceedings of the National Academy of Sciences (USA),* **103**, 10316–10321.

Cleveland CC, Reed SC, Townsend AR (2006) Nutrient regulation of organic matter decomposition in a tropical rain forest. *Ecology,* **87**, 492-503.

Ciarkowska K (2010) Effect of Fertilization on the Structure of Upland Grassland Soil. *Polish Journal of Environmental Studies*, **19**, 693-697.

Crawley MJ (1990) Rabbit grazing, plant competition and seedling recruitment in acid grassland. *Journal of Applied Ecology*, **27**, 803–820.

Defra (2010) *The Fertiliser Manual (RB209).* Department of the Environment, Food and Rural Affairs, TSO (The Stationary Office), London, 252 pp.

del-Val E, Crawley MJ (2005) What limits herb biomass in grasslands: competition or herbivory? *Oecologia*, **142**, 202-211.

De Vries FT, Bardgett R (2012) Plant-microbial linkages and ecosystem nitrogen retention: lessons for sustainable agriculture. *Frontiers in Ecology and the Environment*, **10**, 425-432.

de Vries W, Reinds GJ, Gundersen P, Sterba H (2006) The impact of nitrogen deposition on carbon sequestration in European forests and forest soils. *Global Change Biology,* **12**, 1151–1173.

Edwards GR, Bourdot GW, Crawley MJ (2000) Influence of herbivory, competition and soil fertility on the abundance of *Cirsium arvense* in acid grassland. *Journal of Applied Ecology,* **37**, 321-334.

Elser JJ, Bracken MES, Cleland EE *et al.* (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters,* **10**, 1135–1142.

EUROSTAT (2011) *Agriculture and fishery statistics* - Main results 2009–10. Luxembourg: Publications Office of the European Union.

Farrar J, Hawes M, Jones D, Lindow S (2003) How roots control the flux of carbon to the rhizosphere. *Ecology*, **84**, 827–837.

Fornara DA, Tilman D (2008) Plant functional composition influences rates of soil carbon and nitrogen accumulation. *Journal of Ecology,* **96**, 314-322

Fornara DA, Steinbeiss S, McNamara N *et al*. (2011) Increases in soil organic carbon sequestration can reduce the global warming potential of long-term liming to permanent grassland. *Global Change Biology,* **17**, 1925-1934.

Fornara DA, Tilman D (2012) Soil carbon sequestration in prairie grasslands increased by chronic nitrogen addition. *Ecology,* **93**, 2030–2036.

Harpole WS, Ngai JT, Cleland EE *et al.* (2011). Resource co-limitation of primary producer communities. *Ecology Letters*, **14**, 852-862.

Herencia JF, Garcia-Galavis PA, Maqueda C (2011) Long-term effect of organic and mineral fertilization on soil physical properties under greenhouse and outdoor management practices. *Pedosphere*, **21**, 443–453.

Islam Md R, Chauhan PS, Kim Y, Kim M, Sa T (2011) Community level functional diversity and enzyme activities in paddy soils under different long-term fertilizer management practices. *Biology and Fertility of Soils*, **47**, 599–604.

Janssens IA, Dieleman W, Luyssaert S *et al.* (2010) Reduction of forest soil respiration in response to nitrogen deposition. *Nature Geoscience*, **3**, 315-322.

Kaspari M, Garcia MN, Harms KE *et al.* (2008) Multiple nutrients limit litterfall and decomposition in a tropical forest. *Ecology Letters*, **11**, 35-43.

Liebig J (1840) *Organic Chemistry and its Application to Agriculture and Physiology*. London: printed for Taylor and Walton.

Liu L, Greaver TL (2010) A global perspective on belowground carbon dynamics under nitrogen enrichment. *Ecology Letters*, **13**, 819-828.

Lu M, Zhou X, Luo Y, Yang Y, Fang C, Chen J, Li B (2011) Minor stimulation of soil carbon storage by nitrogen addition: A meta-analysis. *Agriculture Ecosystems and Environment*, **140**, 234–244.

MA - Millennium Ecosystem Assessment - (2005) *Ecosystems and Human Well-being: Synthesis,* World Resources Institute.

Mack MC, Schuur EAG, Bret-Harte MS, Shaver GR, Chapin FS (2004) Ecosystem carbon storage in arctic tundra reduced by long-term nutrient fertilization. *Nature*, **431**, 440-443.

Markgraf W, Watts CW, Richard Whalley W, Hrkac T, Horn R (2012) Influence of organic matter on rheological properties of soil. *Applied Clay Science*, **64**, 25–33.

Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial community in a long-term fertilizer experiment. *Soil Biology & Biochemistry*, **35**, 453–461.

Moharana PC, Sharma BM, Biswas DR, Dwivedi BS, Singh RV (2012) Long-term effect of nutrient management on soil fertility and soil organic carbon pools under a 6-year-old pearl millet–wheat cropping system in an inceptisol of subtropical India. *Field Crop Research*, **136**, 32–41.

Murphy J, Riley JP (1962) A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, **27**, 31-36.

Post WM, Emanuel WR, Zinke PJ, Stangenberger AG (1982) Soil carbon pools and world life zones. *Nature*, **298**, 156–159.

Pregitzer KS, Burton AJ, Zak DR, Talhelm AF (2008) Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. *Global Change Biology*, **14**, 142–153.

R Development Core Team (2008) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Ramirez KS, Craine JM, Fierer N (2012) Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Global Change Biology*, **18**, 1918-1927.

Rodwell, JS (1992) British plant communities. Vol.3. *In: Grasslands and montane communities.* Cambridge University Press, Cambridge, UK.

Six J, Paustian K, Elliott ET, Combrink C (2000) Soil structure and organic matter: I. Distribution of aggregate-size classes and aggregate-associated carbon. *Soil Science Society American Journal*, **64**, 681–689.

Smith BFL, Bain DC (1982) A sodium hydroxide fusion method for the determination of total phosphate in soils. *Communications in Soil Science and Pant Analysis*, **13**, 185-190.

Smith P, Powlson DS, Glendining MJ and Smith JU (1997) Potential for carbon sequestration in European soils: preliminary estimates for five scenarios using results from long-term experiments. *Global Change Biology*, **3**, 67-79.

Smith P, Chapman SJ, Scott WA *et al.* (2007) Climate change cannot be entirely responsible for soil carbon loss observed in England and Wales, 1978-2003. *Global Change Biology,* **13**, 2605-2609.

Smith RS, Shiel RS, Bardgett RD *et al*. (2008) Long-term change in vegetation and soil microbial communities during the phased restoration of traditional meadow grassland. *Journal of Applied Ecology*, **45**, 670–679.

Sprengel C (1828) Von den Substanzen der Ackerkrume und des Untergrundes. *Journal fur Tecnische und Okonomische Chemie,* **2**, 423-474.

Tisdall JM, Oades JM (1982) Organic matter and water-stable aggregates in soils. *European Journal of Soil Science,* **33**, 141–163.

Tong C, Xiao H, Tang G *et al.* (2009) Long-term fertilizer effects on organic carbon and total nitrogen and coupling relationships of C and N in paddy soils in subtropical

China. *Soil & Tillage Research*, **106**, 8–14.

Townsend AR, Cleveland CC, Houlton BZ, Alden CB, White WC (2011) Multi-nutrient regulation of the tropical forest carbon cycle. *Frontiers in Ecology and the Environment*, **9**, 9-17.

Trumbore SE, Gaudinski JB (2003) The secret lives of roots. *Science,* **302**, 1344–1345.

Viets FGA (1977) Perspective on Two Centuries of Progress in Soil Fertility and Plant Nutrition. *Soil Science Society American Journal*, **41**, 242–249

Yu H, Ding W, Luo J, Geng R, Ghani A, Cai Z (2012) Effects of long-term compost and fertilizer application on stability of aggregate-associated organic carbon in an intensively cultivated sandy loam soil. *Biology and Fertility of Soils*, **48**, 325–336.

Fig. 1. Dependence of total soil C pools (0-20 cm depth) on different nutrient fertilization treatments. Legend: All = all nutrients applied (N, P, K, Mg); Control = no nutrients; N = only N; P = only P; NP = NP together; K = only K; PK = PK together. Error bars show ±SE and represent variation among plots receiving the same nutrient addition treatment.

Fig. 2. Dependence of total soil C pools (0-20 cm depth) on different nutrient additions in the presence/absence of lime applications (a), and rabbit grazing (b). Error bars show ±SE and represent variation among plots receiving the same nutrient addition treatment.

Fig. 3. Dependence of soil "C gain efficiency" (i.e. units of soil C gained per units of N added; a, b) and "N retention efficiency" (i.e. units of soil N retained per units of N added; c, d) on the addition of P or K nutrients. On x-axes, "N = No" are plots receiving N-only, and "Y = Yes' plots receiving either P or K in combination with N.

Fig. 4. Dependence of total plant aboveground biomass (a), and of proportion of biomass of different plant functional groups (b) on different nutrient fertilization treatments. Plant aboveground biomass was measured in 1997.

Fig. 5. Effects of different nutrient fertilization treatments on plant species diversity (a), and on relative plant aboveground cover (i.e. proportion cover) of different functional groups (b). Proportion cover and number of plant species were estimated in 2012.

Fig. 6. Dependence of total root mass on different nutrient fertilization treatments. Total root mass was measured in 2011.

Fig. 7. Effects of nutrient treatments on soil N losses (a), and soil P losses (b). To estimate soil N and P losses we first calculated the difference in total soil N (and P) pools between each nutrient-fertilized plot and the average of the controls in 2011 and then subtracted this value from the total amount of N or P added over 19 years.

Fig. 1

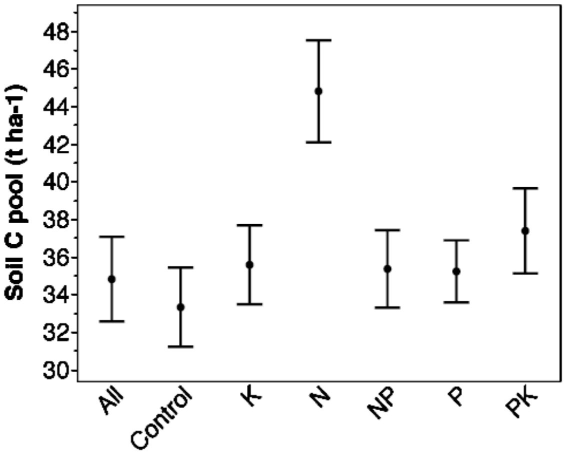
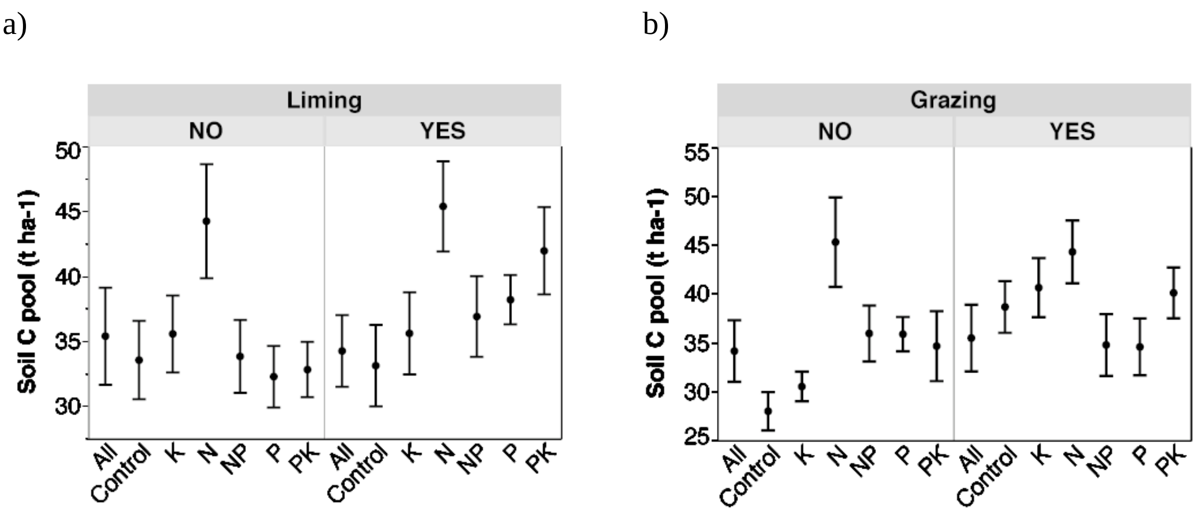

Soil C pool (t ha-1)
48
46
44
42
40
38
36
34
32
30
All
Control
K
N
NP
P
PK

Fig. 2


a)

b)

Liming
NO
YES
Soil C pool (t ha-1)
50
45
40
35
30
All
Control
K
N
NP
P
PK
Grazing
NO
YES
Soil C pool (t ha-1)
55
50
45
40
35
30
25
All
Control
K
N
NP
P
PK

Fig. 3

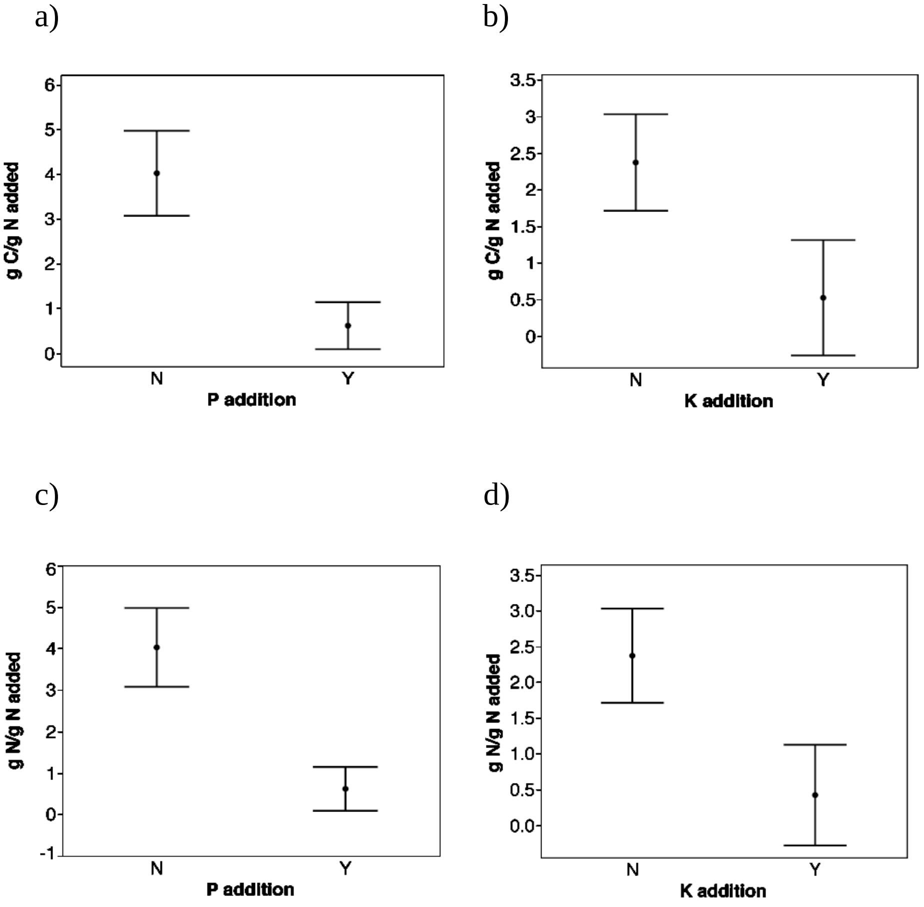

a)
b)
c)
d)
g C/g N added
P addition
K addition
g N/g N added
N
Y
0
1
2
3
4
5
6
-1
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

Fig. 4

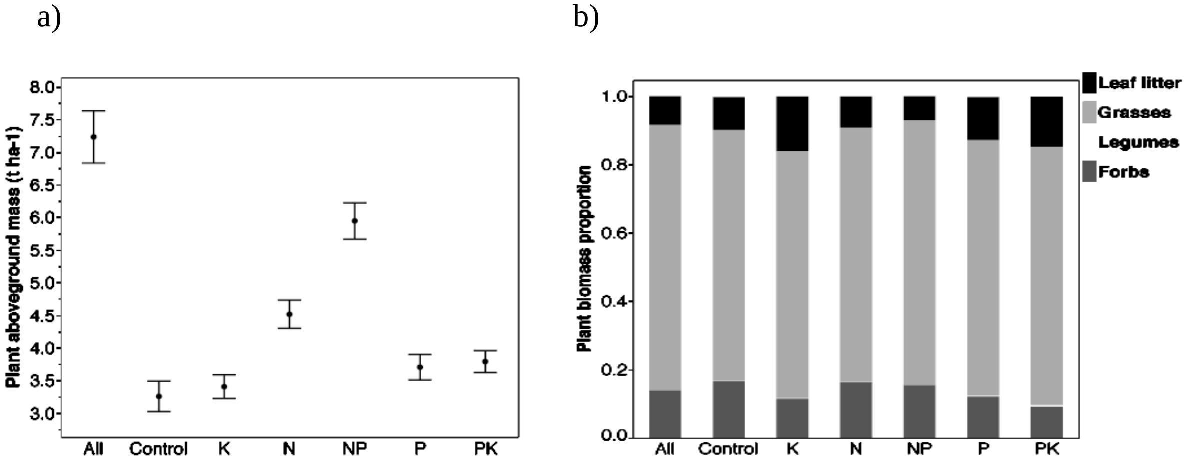
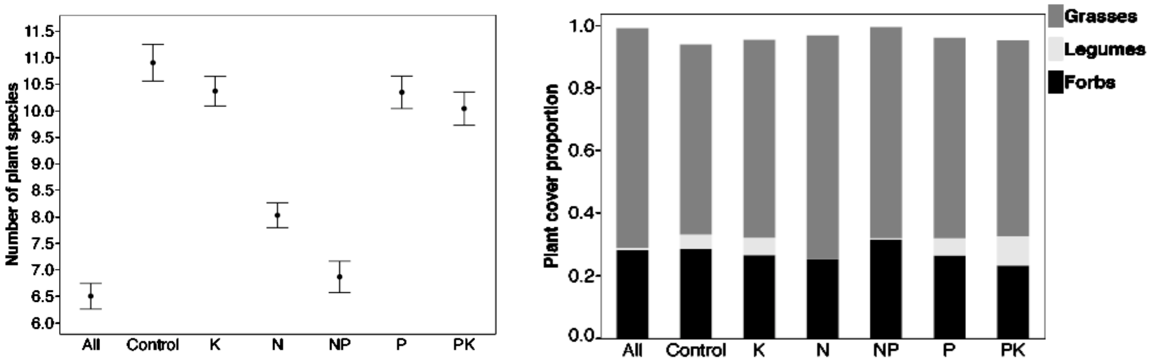

a)
b)
Plant aboveground mass (t ha-1)
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
All
Control
K
N
NP
P
PK
Plant biomass proportion
0.0
0.2
0.4
0.6
0.8
1.0
Leaf litter
Grasses
Legumes
Forbs

Fig. 5


a)

b)

Fig. 6

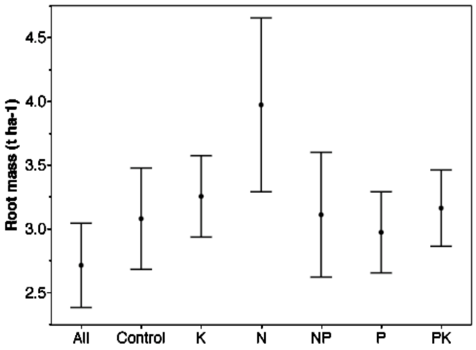
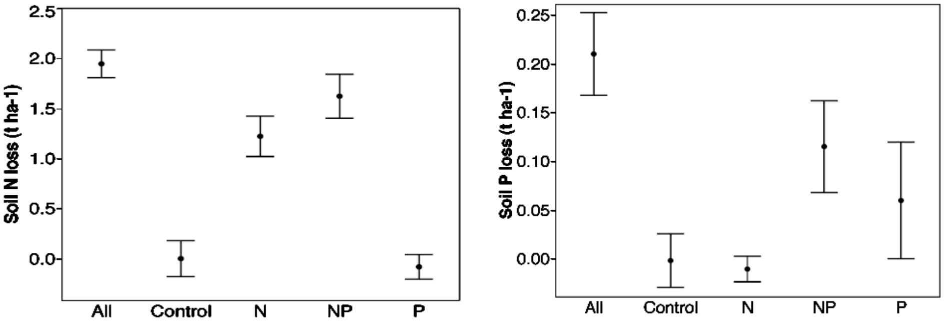


Fig. 7


a)

b)

Supplementary material

Fig. 1. Experimental design of Nash's Field. We used four large 45m x 20m plots, two of them randomly selected receiving insecticide (–insects) and two plots with no insecticide application (+insects). Within each of these large plots the split plot design is shown as an example. The position of grazing, liming and nutrient treatments was randomized within each set of sub-plots. Four mineral nutrients were applied to the 2m×2m plots as following: nitrogen (N) (as ammonium nitrate) at 100 kg N ha^{-1}, phosphorus (P) (as triple super-phosphate) at 35kg P ha^{-1}, potassium (K) (as muriate of potash) at 225 kg K ha^{-1} and magnesium (Mg) (as magnesium sulphate) at 11 kg Mg ha^{-1}. Legend: All = all nutrients applied (N, P, K, Mg); Control = no nutrients; N = N only applied; P = only P; NP = NP together; K = only K applied; PK = PK together.

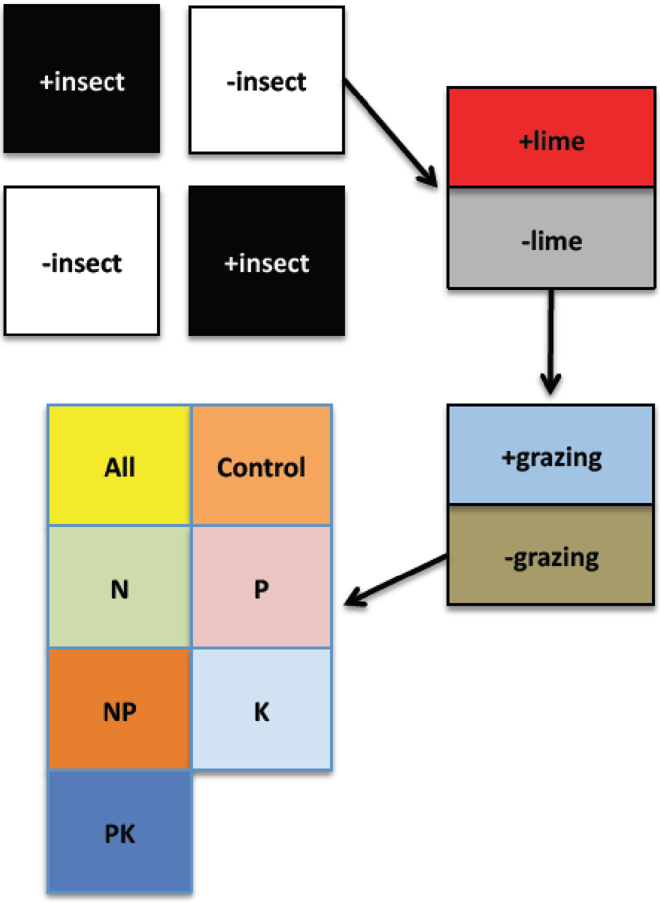


Fig. 2. Dependence of total soil N pool on different nutrient addition treatments. Error bars show ±SE and represent variation among plots receiving the same nutrient addition treatment.

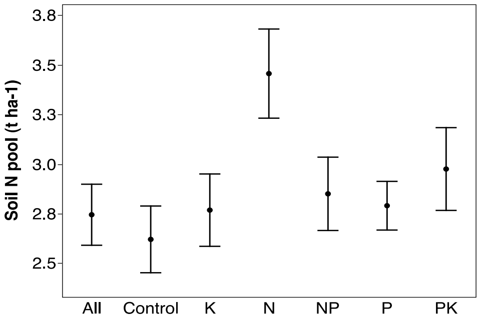
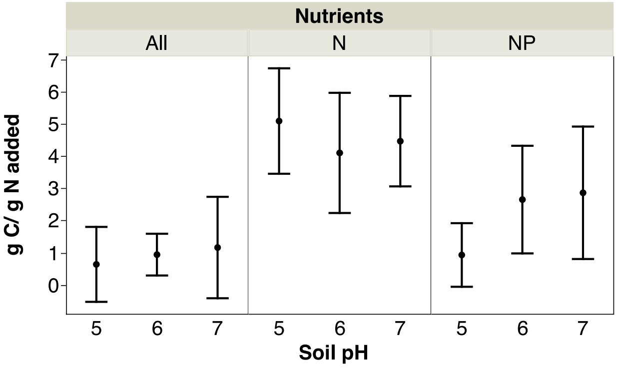


Fig. 3. Dependence of soil "C gain efficiency" on different N-additions (All = all nutrients added, N = N only, NP = NP together) across a broad range of soil pH values: "5" = pH<5; "6" = 6<pH<7; "7" = pH>7 (a). Also variation in soil pH under liming (b) and grazing treatments (c) across different nutrient additions.

a)



b)

c)

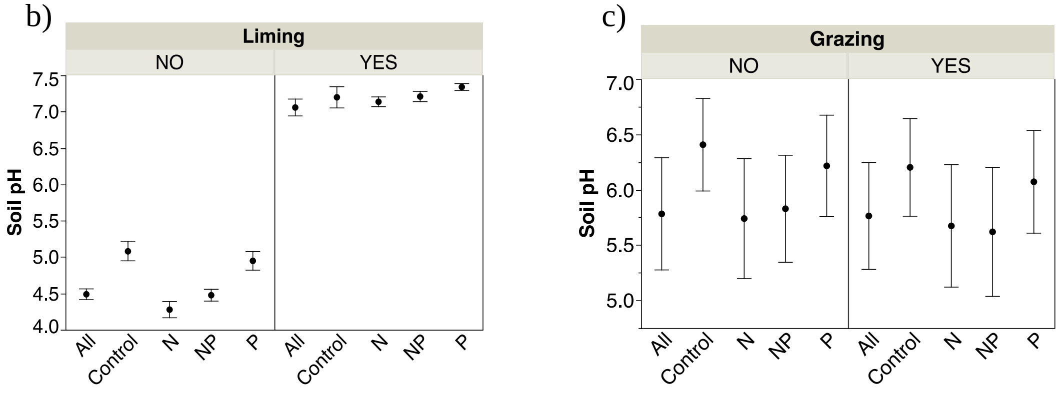


Fig. 4. Dependence of total root C and N pools on different nutrient treatments.

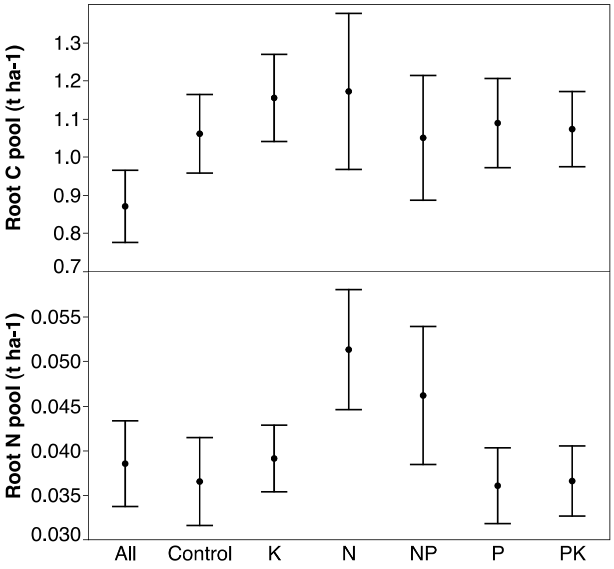


Fig. 5. Dependence of soil C:N:P stoichiometry (a), and root C:N:P stoichiometry (b) on different nutrient treatments. Soil stoichiometry was not measured in the K and PK treatments.

a) b)

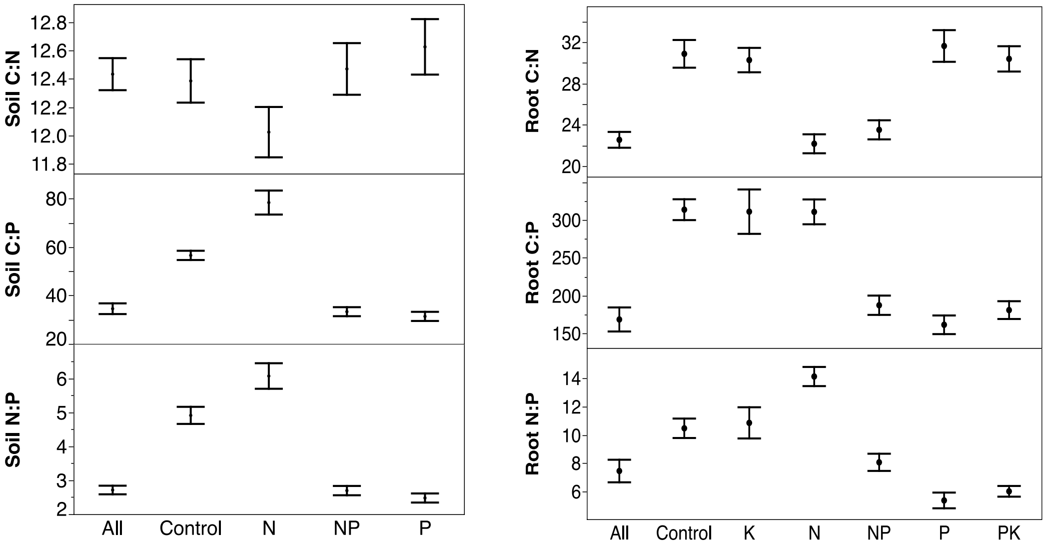


Fig. 6. Significant relationship between root C:N ratios and soil C pools ($F_{1,110} = 14.9$, $P = 0.0002$). The gray band around the regression line represents 95% confidence intervals.

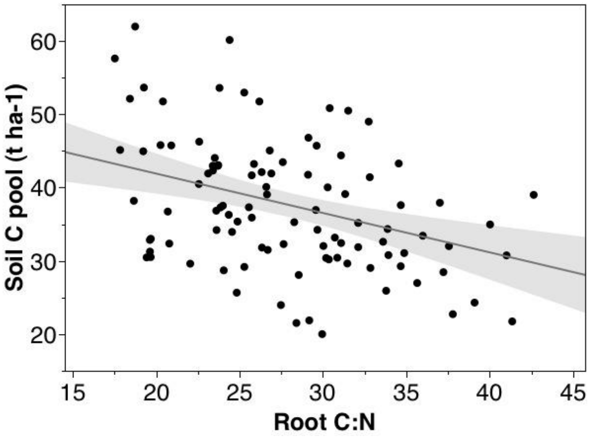


Fig. 7. Dependence of net soil N mineralization rates on different nutrient additions in the presence/absence of grazing disturbance (a), and liming applications (b). Error bars show ±SE and represent variation among plots receiving the same nutrient addition treatment.

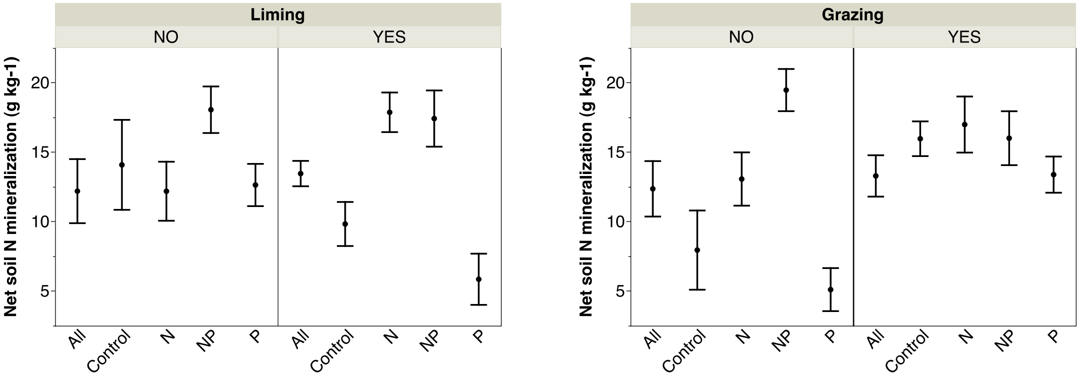


Table 1. An example output of our mixed effects ANOVA models. Parameter estimates for final model for the response variable total soil C pool (t C ha^{-1}), where 'nutrient treatment' is a fixed factor effect and 'block' is a random effect, using the syntax y ~ Nutrients + (1|Block). Between-factor level differences were subsequently examined using Tukey *post-hoc* tests.

Fixed effects Nutrient treatment	Estimate	SE	t-value
All	34.80	2.87	12.11
Control	33.31	2.78	-0.54
K	35.57	2.78	0.27
N	44.79	2.78	3.59
NP	35.35	2.78	0.20
P	35.21	2.78	0.15
PK	37.37	2.78	0.92
Random effects standard deviation: 4.19			
Residual standard deviation: 7.88			