Reconciling multiple impacts of nitrogen enrichment on soil carbon: plant, microbial and geochemical controls

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Abstract
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Keywords
acidification, calcium bridging, density fractionation, mineral sorption, nitrogen enrichment, nitrogen limitation

Disciplines
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Statement of authorship

SH and YB conceived the research. YB and DC designed and conducted the field experiment. CY and DC performed the lab analyses. CY, DC, SH, SJH and YB analyzed the data. CY, SJH, and SH wrote the manuscript with significant inputs from all other authors.

Data accessibility statement

We confirm that if our manuscript is accepted, the data supporting the results will be archived in an appropriate public repository.
Abstract

Impacts of reactive nitrogen (N) inputs on ecosystem carbon (C) dynamics are highly variable, and the underlying mechanisms remain unclear. Here, we proposed a new conceptual framework that integrates plant, microbial, and geochemical mechanisms to reconcile diverse and contrasting impacts of N on soil C. This framework was tested using long-term N enrichment and acid addition experiments in a Mongolian steppe grassland. Distinct mechanisms could explain effects of N on particulate and mineral-associated soil C pools, potentially explaining discrepancies among previous N addition studies. While plant production predominated particulate C changes, N-induced soil acidification strongly affected mineral-associated C through decreased microbial growth and pH-sensitive associations between iron and aluminum minerals and C. Our findings suggest that effects of N-induced acidification on microbial activity and geochemical properties should be included in Earth system models that predict ecosystem C budgets under future N deposition/input scenarios.
INTRODUCTION

Anthropogenic reactive nitrogen (N) inputs, largely caused by fossil-fuel combustion and chemical fertilizer applications, have exceeded N inputs through all natural processes over recent decades (Vitousek et al. 1997; Davidson 2009). Because of the tight coupling between terrestrial carbon (C) and N cycles, the increased N in soil has considerably altered terrestrial ecosystem processes such as plant productivity and decomposition, that in turn affect ecosystem C dynamics (Reay et al. 2008; Liu & Greaver 2010; Riggs & Hobbie 2016). Soils contain the largest near-surface reservoir of organic C in terrestrial ecosystems (Jobbagy & Jackson 2000), and changes in soil C in response to N enrichment may thus have substantial impacts on the net CO₂ exchange between the atmosphere and the biosphere.

The impact of N inputs on soil C storage has received considerable research attention. In theory, N inputs can stimulate the production of plant biomass, increasing C inputs to soil (Lebauer & Treseder 2008; Hyvönen et al. 2008; Pregitzer et al. 2008; Fornara & Tilman 2012). However, many studies have shown that increased net primary production does not always lead to net increases in C storage. Neutral and negative effects of N inputs on soil C storage have also been reported (Neff et al. 2002; Mack et al. 2004; Zeglin et al. 2007; Liu & Greaver 2010).

A key source of the inconsistent and conflicting results among studies may lie in the differential responses of various soil C pools to N enrichments (Averill & Waring 2017). The total soil C stock can be represented by multiple operational pools with
different turnover times linked to physico-chemical protection mechanisms (Christensen 2001; Zimmermann et al. 2007). Researchers often separate total soil C into light and heavy fractions by density fractionation (von Lützow et al. 2007). Light fraction C consists of partially decomposed plant materials and by-products of decomposition (and potentially charred material), while heavy fraction C consists of mineral-sorbed C and a variable fraction of aggregate-occluded particulate C (Six et al. 2006; von Lützow et al. 2007). These density fractions represent mixtures of pools with differing turnover times (Torn et al. 2013; Hall et al. 2015), but nonetheless represent useful operational separations for understanding soil C cycling processes (Christensen 2001; Zimmermann et al. 2007).

Intriguingly, the effects of N addition on both light and heavy fraction C differ among studies. The impact of N addition on light fraction C has been reported to be both positive (Huang et al. 2011; Song et al. 2014) and negative (Neff et al. 2002; Cusack et al. 2011). Similarly, many field experiments have shown that N additions promoted soil C accumulation in mineral-associated fractions (Neff et al. 2002; Cusack et al. 2011; Huang et al. 2011), but contradicting results have also been reported (Bradford et al. 2008; Song et al. 2014; Riggs et al. 2015). Multiple microbial mechanisms have been proposed to explain these controversial results, such as changes in microbial extracellular enzyme activities, the decomposer community, or microbial carbon use efficiency under N additions (Fog 1988; Schimel & Bennett 2004; Waldrop et al. 2004; Riggs & Hobbie 2016), but the exact mechanisms remain
uncertain. While N addition increases N availability, it can also cause soil acidification. This is due to increased nitrification and associated proton (H⁺) production accompanied by leaching of base cations with nitrate (NO₃⁻), as well as the biological uptake of ammonium (NH₄⁺), which also releases H⁺ (Fig. 1; Matson et al. 1999; Guo et al. 2010). At the global scale, soil pH decreases linearly with N addition (Tian & Niu 2015), consistent with an exponential increase in [H⁺]. Soil acidification can strongly affect microbial metabolism. Addition of N tends to decrease microbial biomass (Liu & Greaver 2010; Treseder 2008) and a recent N addition experiment showed that soil acidification best explained observed decreases in microbial biomass and microbial respiration (Chen et al. 2016). Emerging evidence has also demonstrated that microbial residues derived from catabolism of light fraction C are important constituents contributing to mineral-associated C (Cotrufo et al. 2015; Liang et al. 2017). Thus, regardless of increased plant litter inputs, N-induced acidification may suppress microbial decomposition and hence decrease the pool of microbial biomass that serves as precursors for C inputs to heavy fractions (i.e., organo-mineral associations).

In addition, increased leaching of base cations (e.g., K⁺, Mg²⁺ and Ca²⁺) and greater solubility of iron (Fe) and aluminum (Al) phases that co-occur with acidification could also play a key role in altering the sorption and co-precipitation reactions that affect mineral-associated C. Some studies found that the increased
solubility of hydrolyzing cations (e.g., Fe$^{3+}$ and Al$^{3+}$) at low pH could increase the stabilization of C in organo-metal associations thought to protect C from microbial decomposition (Gu et al. 1994; Mueller et al. 2012). Most organo-metal complexes associate with the heavy, mineral-associated fraction (Kaiser & Guggenberger 2007). However, losses of polyvalent cations—especially Ca$^{2+}$—can promote the release of organic C adsorbed to mineral surfaces through polyvalent cation bridging (von Lützow et al. 2006). Thus, the effects of N additions on soil C pools depend not only on direct effects on plant productivity and microbial activity, but also on soil geochemical properties mediated by N-induced soil acidification.

The interrelated responses of plants, microbes and minerals to N inputs likely result in contrasting effects on different soil C pools. Although a model simulation recently showed that microbial physiology, mineralogy, and acidity may co-affect the impact of N inputs on soil C dynamics (Averill & Waring 2017), no experimental studies to date have directly demonstrated these interactions in field. In this study, we propose and test a conceptual framework linking multiple plant, microbial, and geochemical mechanisms to explain the variable and potentially counteracting effects of N addition on soil C pools (Fig. 1). First, N inputs directly increase N availability for plants and soil microbes. In N-limited ecosystems, this increase in soil N availability could promote plant growth and increase production of plant litter (Lebauer & Treseder 2008). Although the increase in quantity of plant biomass could increase light fraction C, this would depend on concomitant effects of N on litter
decomposition rates. Therefore, the change of soil labile C (largely particulate C) in response to N addition depends on both production of plant biomass and soil microbial activity. Second, N inputs can also result in soil acidification (Guo et al. 2010; Chen et al. 2016), thereby suppressing microbial decomposition, increasing the solubility of Fe and Al oxides, and enhancing leaching of base cations such as Ca$^{2+}$. Thus, acidification may disrupt C that is sorbed via Ca bridging, while simultaneously increasing pools of C co-precipitated with Fe and Al phases. We hypothesize that the relative strengths of these biotic and abiotic responses will determine the net effect of N addition on organo-mineral associations.

Taking advantage of a long-term N enrichment experiment initiated in 1999, we examined the effects of a gradient on N inputs on soil C pools in a Mongolian steppe grassland. This biome forms part of the Eurasian Steppe, extending over 8000 km from north-eastern China to Hungary (Bai et al. 2007). To assess impacts of N-mediated acidification, an acid addition experiment was conducted adjacent to the N enrichment experiment to generate comparable decreases in soil pH resulting from N inputs (Chen et al. 2016). This allows us to compare the effects of N-induced soil acidification and direct acid additions on soil C pools. Our objectives were to 1) investigate the effects of long-term reactive N inputs on various C pools with an emphasis on reconciling impacts of N on plant productivity, microbial decomposition, and C partitioning within and among density fractions, and 2) ascertain the role of N-induced acidification in regulating soil C dynamics.
MATERIAL AND METHODS

Study site

This study was conducted at the Inner Mongolia Grassland Ecosystem Research Station of the Chinese Academy of Sciences, which is located in the Xilin River Basin of Inner Mongolia, China (43°38' N, 116°42' N and 1250 m a.s.l.). The semi-arid continental climate characterizes this steppe ecosystem. The mean annual temperature is 0.3 °C, with mean monthly temperatures ranging from -21.6 °C in January to 19.0 °C in July. Mean annual precipitation is 346.1 mm, with about 80 % falling in the growing season (June–August). The soil at this site is classified as dark chestnut (Calcic Chernozem according to ISSS Working Group RB, 1998), with a loamy-sand texture. The clay, silt and sand contents were 2.22 %, 17.60 % and 80.18 %, respectively (Bai et al. 2010).

Field experiments

The N enrichment experiment was established in 1999. There were six levels of N addition rate: 0, 1.75, 5.25, 10.50, 17.5, and 28.0 g N m\(^{-2}\) yr\(^{-1}\). Fifty-four plots each measuring 5 × 5 m were arranged in a randomized block design including nine replicate blocks and separated by 1 m buffers. Reactive N was added as commercial pelletized NH\(_4\)NO\(_3\) fertilizer at the middle of the growing season (July 1 – 5). To ensure that N was the primary limiting nutrient, each plot also received P (10 g P\(_2\)O\(_5\) m\(^{-2}\) yr\(^{-1}\)), S (0.2 mg m\(^{-2}\) yr\(^{-1}\)) and trace elements (Zn: 190 μg m\(^{-2}\) yr\(^{-1}\), Mn: 160 μg m\(^{-2}\) yr\(^{-1}\)).
yr\(^{-1}\), B: 31 \(\mu g\) m\(^{-2}\) yr\(^{-1}\)). Because these soils were rich in K, Mg, Ca and Fe, these four nutrients were not added in this experiment (Bai et al. 2010). We added P and other micronutrients at a constant level across N addition treatments to ameliorate co-limitation by these nutrients in our system. This should be noted when comparing our results to other N addition experiments where limitation by multiple nutrients may have occurred, thus influencing the plant and microbial responses to N addition.

To assess the potential direct effects of soil acidification on soil organo-mineral associations, a complementary acid addition experiment was established near the N enrichment experimental site in 2009 to generate comparable degrees of soil acidification resulting from N inputs (Chen et al. 2016). There were seven levels of acid addition: 0, 2.76, 5.52, 8.28, 11.04, 13.80 and 16.56 mol H\(^+\) m\(^{-2}\) yr\(^{-1}\) (a detailed description is provided in Materials and Methods S1).

**Plant and soil sampling**

In this study, we chose four treatments from the N enrichment experiment (0, 1.75, 10.50 and 28.0 g N m\(^{-2}\) yr\(^{-1}\)) and four treatments from the acid addition experiment (0, 2.76, 11.04 and 16.56 g mol H\(^+\) m\(^{-2}\) yr\(^{-1}\)) for plant and soil sampling. In August 2014, above-ground vegetation was collected in a 0.5 m \(\times\) 0.5 m quadrat to determine above-ground plant biomass in each plot of the N enrichment experiment and acid addition experiment. After the above-ground biomass was sampled, three soil cores (6.5 cm diameter and 0–20 cm depth) were collected in each plot to determine below-ground plant biomass. Then four soil cores (2 cm diameter, 0–20 cm depth) were
randomly collected from each selected plot and combined to form one composite soil sample. After roots and rocks were removed, the field moist soil was passed through a 2 mm mesh sieve and thoroughly mixed. The fresh soil was used for determination of microbial parameters. Air-dried samples were used for determination of physicochemical properties (Materials and Methods S1).

**Determination of soil microbial biomass and microbial activity**

Soil microbial biomass was estimated using the chloroform fumigation-extraction method (Vance *et al.* 1987). Briefly, 12.5 g of fresh soil was fumigated with ethanol-free chloroform for 48 h. Both fumigated and non-fumigated soils were extracted with 50 mL 0.5 M K$_2$SO$_4$ solution and the extracts were measured on the TOC analyzer to determine organic C concentrations. The measured organic C was converted to microbial biomass C using a conversion factor $k_{ec} = 0.33$ (Mo *et al.* 2008). Soil microbial activity was measured by determining microbial respiration during an 11-week incubation period. The respired CO$_2$ was determined by an incubation-alkaline absorption method (Hu & vanBruggen 1997). The cumulative C lost by respiration during incubation was calculated following the formula described by Liu *et al.* (2009).

**Density fractionations and their C contents**

The bulk soil was separated into two operationally defined soil fractions, a free light fraction and a heavy fraction (> 1.8 g cm$^{-3}$), following a density fractionation technique. Then, the separated soil fractions were dried in an oven at 80 °C and then ground to a homogenized fine powder for organic C analysis using the CN analyzer.
(Materials and Methods S1).

**Determination of Ca-bound C, Fe-bound C, and Fe and Al phases**

The heavy fraction soil samples were sequentially extracted with 0.5 M Na$_2$SO$_4$ and dithionite-citrate-bicarbonate (DCB; soil:solution ratios of 1:50 for both extractions) to separate organic C associated with Ca bridges and Fe mineral phases, respectively. Because water soluble and weakly-exchangeable organic matter were removed from soil colloids during the NaI density separation and subsequent rinses, we interpret C released during Na$_2$SO$_4$ extraction as predominantly representing organic C associated with Ca bridges (Xu & Yuan 1993). These solutions were measured on the TOC analyzer. Following Na$_2$SO$_4$ extraction, the residual soil was extracted in DCB to release Fe-associated organic C (Materials and Methods S1).

**Determination of soil total crystalline and short-range-ordered minerals**

Total mineral masses of short-range-ordered (SRO) and crystalline phases were estimated by sequential chemical extractions of the heavy soil fractions (Chadwick *et al.* 2003). The extraction procedures were as follows: (1) Organic matter was removed with hydrogen peroxide and then the residual soil was rinsed and air dried. (2) The residue was extracted using 0.275 M ammonium oxalate (pH 3.25) in the dark and non-crystalline minerals were determined by recording weight loss after extraction. (3) The residue from the ammonium oxalate dissolution was extracted by the DCB method described above and weighed to determine crystalline mineral content. The contents of SRO and crystalline minerals were expressed on a total soil basis.
Statistical analyses

First, we used a linear mixed effects model to assess the impacts of N or acid additions on response variables, with N or acid treatments as fixed effects and blocks as a random effect. Then, one-way ANOVA with Duncan's multiple-range tests was performed across all response variables to compare the effects of N or acid additions. Second, we performed linear regression to analyze the responses of soil microbial biomass C, CO₂ efflux, total C in heavy fraction, Ca-bound C and Fe-bound C to soil pH. We also performed linear regression to analyze the responses of organic C in light fraction to plant biomass for N enrichment and acid addition experiment. In addition, ANCOVA was performed to estimate the slopes of these linear regressions and compare their differences between N enrichment experiment and acid addition experiment. All statistical analyses were performed using R version 3.3.3.

A structural equation modelling (SEM) approach was used to test a conceptual model for N enrichment impacts on soil particulate C and mineral-associated C (Fig. 1). We used principal component analysis to create a multivariate index for NH₄⁻-N and NO₃⁻-N. The first principal component, which explained 91.0 % of the total variance, was used in the subsequent SEM analysis. The SEM analysis was performed with the lavaan package (Rosseel 2012) in R version 3.3.3 using the maximum likelihood estimation method. Several tests were used to assess model fit: the χ²-test, comparative fit index (CFI), and root square mean error of approximation (RMSEM).
RESULTS

Soil abiotic properties, plant biomass and microbial biomass and respiration

Both N inputs and acid addition significantly decreased soil pH, with similar decreases evident in both experiments ($P < 0.05$, Table S1). However, they had contrasting effects on several C and N metrics. While N inputs increased soil NO$_3^-$-N, NH$_4^+$-N, total organic C, and total N, acid additions reduced all of these metrics ($P < 0.05$, Table S1). Neither N addition nor acid addition had significant impacts on dissolved organic C (Table S1).

Addition of N significantly increased both above-ground and below-ground plant biomass ($P < 0.05$, Fig. 2a, b), but significantly reduced microbial respiration and microbial biomass C ($P < 0.05$, Fig. 3a, b). Acid additions had similar effects as N additions on microbial respiration and microbial biomass C ($P < 0.05$, Fig. 3c, d), but had no effects on plant biomass (Fig. 2c, d). Specifically, the cumulative microbial respiration and microbial biomass C decreased with soil pH for both experiments, and the slopes and intercepts of the linear regressions between pH and the two microbial parameters did not significantly differ between the two experiments, except for the intercepts between pH and microbial respiration ($P < 0.05$, Fig. 3e, f).

Soil organic C in different fractions

Addition of N significantly increased light fraction C ($P < 0.05$, Fig. 4a), while acid addition significantly decreased light fraction C ($P < 0.05$, Fig. 4b). In the N enrichment experiment, increases in above-ground and below-ground plant biomass
explained 51 % and 39 % of the variation in light fraction C, respectively (Fig. 4c, d).

In comparison, plant biomass had no significant effect on light fraction C in the acid addition experiment (Fig. 4c, d).

Both N inputs and acid addition significantly reduced the content of heavy fraction C and Na2SO4-extractable C (interpreted as Ca-bound C), but increased dithionite-extractable (interpreted as Fe-bound) C ($P < 0.05$, Fig. 5a–f). Linear regressions showed that decreased soil pH was significantly related to decreases in total heavy fraction C and the Ca-bound component of heavy fraction C, and increases in Fe-associated component of heavy fraction C, for both experiments (Fig. 5g–i). The slopes of the linear regressions between pH and these three response variables (heavy fraction C, Ca-bound C, and Fe-associated C) did not significantly differ between the N addition and acid addition experiments (Fig. 5g–i).

**Geochemical characteristics**

Neither N nor acid addition had significant impacts on DCB-extractable Fe and Al, SRO Fe and Al, total SRO minerals, and crystalline minerals (Table S1; Fig. S1). However, both N and acid additions significantly increased pyrophosphate-extractable Fe and Al concentrations ($P < 0.05$, Table S1), and decreased extractable Na+, K+, Mg²⁺, Ca²⁺, and Mn²⁺ concentrations ($P < 0.05$, Fig. S2), consistent with our conceptual framework (Fig. 1).

**Controls on light and heavy fraction C in the N addition experiment**

The optimal SEM model implied by our data supported most of the hypothesized
relationships among biogeochemical variables and soil C fractions (Fig. 6). Addition of N directly induced changes in soil N availability and pH, explaining 93 % and 94 % of the total variance in these two variables, respectively (Fig. 6). Soil N availability directly explained 73 % of the variance in above- and belowground biomass, which in turn was associated with increased particulate organic C ($R^2 = 49 \%$). However, the decreased soil pH resulting from N addition (as opposed to N availability per se) was the optimal predictor for several response variables. Decreased pH was associated with decreases in microbial biomass and Ca-associated C, and increased Fe-associated C ($R^2$ values between 56 and 74 %). These findings were in accordance with the results of the simple linear regressions described above (Fig. 3f; Fig. 5h, i), and indicated that soil acidification induced by N addition directly impacted microbial metabolism and mineral-associated C. In addition, particulate organic C had a direct negative relationship with mineral-associated organic C, while soil microbial biomass, and Fe- and Ca-associated C were not significantly related to mineral-associated organic C.

**DISCUSSION**

Our study showed that although long-term N inputs significantly increased plant litter production and bulk soil organic C, they significantly reduced heavy fraction C content. Soil pH showed a strong, linear decrease with N addition, consistent with global observations (Tian & Niu 2015). Consistent with our proposed conceptual
framework, our results suggested that the reduced heavy fraction C was largely
induced by soil acidification following N addition, which was confirmed by our
complementary acid addition experiment and linear regressions (Fig. 5a, d, g). In
addition, our study also demonstrated that N-induced soil acidification led to
significant increased content of Fe-bound C and reduced content of Ca-bound C.
Together, these findings suggest that soil acidification may control impacts of N on
soil C dynamics via contrasting biological and geochemical mechanisms.

**Nitrogen enrichment increased total soil C and light fraction C pools**

Results from our study showed that long-term N additions enhanced total soil
organic C by increasing the C mass in the light fraction (Table S1). Enhancement of
soil organic C by N fertilization has been reported in many previous studies (e.g.
Fornara & Tilman 2012; Yue et al. 2016) and has been largely attributed to N
stimulation of plant growth and subsequent organic C inputs via aboveground
residues and roots (Lebauer & Treseder 2008; Liu & Greaver 2010). We also found
that N addition significantly increased light fraction C stocks (Fig. 4a), in contrast to
Neff et al. (2002) and Cusack et al. (2011) who found that N additions did not affect
light fraction C and reduced light fraction C, respectively. Previous studies proposed
that enhanced decomposition of light fraction C with added N resulted from
increasing labile C inputs to soils (i.e., the priming effect), alleviating N limitation of
microbes, and/or facilitating the production of extracellular enzymes (Schimel &
Bennett 2004; Cusack et al. 2011; Riggs et al. 2015). In our study, N enrichment
increased both above- and below-ground biomass, consistent with the findings of a meta-analysis (Xia & Wan 2008). Our statistical models (Fig. 6) implied that the increase in light fraction C with N addition was largely due to increased plant biomass inputs, as evidenced by a strong positive relationship between above- and below-ground biomass and light fraction C (Fig. 4c, d). In the acid addition experiment, we found a small but significant decrease in light fraction C with increasing acidity even though plant biomass was unaffected in 2014. This may have resulted from a slight suppression of plant productivity under acid addition that occurred earlier in the experiment (Chen et al. 2013).

**Nitrogen enrichment decreased the heavy fraction C pool**

Contrary to increases in heavy fraction C observed in some other N addition studies (Neff et al. 2002; Huang et al. 2011; Cusack et al. 2011), we found that N additions significantly reduced heavy fraction C (Fig. 5a). The net amount of heavy fraction C depends on the balance between newly transformed C from faster-cycling pools and decomposition losses from the existing heavy fraction C. The majority of heavy fraction C in temperate ecosystems often cycles over decadal timescales (Baisden et al. 2002; Koarashi et al. 2012), consistent with our observations of significantly altered heavy fraction C stocks following 15 years of N addition. Since N additions had consistently increased plant production and litter inputs over time in our study site (Bai et al. 2010; Chen et al. 2015; Chen et al. 2016), the decreased heavy fraction C was likely due to reduced organic C inputs to the decadal-cycling heavy fraction pool.
and/or increased decomposition losses of the existing heavy fraction C. Therefore, we propose that at least two mechanisms contributed to this decrease, consistent with our statistical models and comparisons between findings from the N addition and acid addition experiments.

First, decreases in microbial biomass were statistically associated with strong decreases in heavy fraction C (Fig. S3), consistent with the importance of microbial detritus as a key input to mineral-associated C pools (Cotrufo et al. 2013, Lehmann & Kleber 2015). It has long been noted that N can alter microbial enzyme production, potentially contributing to suppression of lignin-degrading enzymes while potentially enhancing carbohydrate catabolism (Fog 1988). We found that suppression of microbial biomass and respiration under N addition were strongly linked to decreased pH, as confirmed by the similar slopes of the linear regressions between pH and microbial respiration and biomass for the N enrichment and acid addition experiments (Fig. 3e, f). Addition of N had an additional suppressive effect on microbial respiration indicated by differing regression intercepts between the N and acid addition experiments (Fig. 3e), but intercepts for microbial biomass were similar (Fig. 3f). These findings strongly suggested that acidification, not N addition per se, drove the decrease in microbial biomass with pH. Furthermore, the SEM analyses were consistent with direct effects of acidification following N inputs, rather than the increased soil N availability itself, in suppressing microbial biomass (Fig. 6).

Microbes can further influence mineral-associated C fluxes by affecting soil
structure. Fungal hyphae and plant roots often promote aggregate formation, which can further protect mineral-associated C from microbial attack (Wilson et al. 2009). At our experimental site, recent studies observed that long-term N additions significantly reduced mycorrhizal and total fungal biomass (Chen et al. 2016; Wei et al. 2013). Together, these results indicate that N-induced reduction in microbial biomass and activities, likely mediated by acidity, could reduce both C inputs to the heavy fraction and their physical protection via aggregation.

The second likely mechanism contributing to decreased mineral-associated C was the replacement of Ca\(^{2+}\) by H\(^{+}\) and the subsequent leaching of Ca\(^{2+}\) along with nitrate, and release of C stabilized via cation bridging. To the best of our knowledge, this mechanism has not previously been addressed in the context of N impacts on ecosystem C dynamics, but is likely to be important in many soils with circumneutral pH where significant C is stabilized via Ca bridging (Oades 1988). The vast majority of studies have so far attempted to explain the impact of N additions on soil C dynamics by focusing on litter inputs and microbial decomposition (Liu & Greaver 2010; Janssens et al. 2010; Lu et al. 2011) rather than geochemical mechanisms of C stabilization. For example, Cusack et al. (2011) attributed increased heavy-fraction C in response to N addition to decreases in microbial oxidative enzymes. However, long-term N additions can induce a series of changes in soil physical and chemical properties, particularly soil acidity (Guo et al. 2010), which may directly affect mineral-organic C interactions. Emerging evidence has recently shown that protection
of organic C from microbial attack, rather than intrinsic organic matter recalcitrance, plays a dominant role in maintaining soil organic C stability (Schmidt et al. 2011; Lehmann & Kleber 2015). Organo-mineral complexes can act as nuclei to form microaggregates that are key to organic C protection (Vogel et al. 2014; Yu et al. 2017). Soil acidification can affect the speciation and solution activities of soil minerals and cations that interact with organic carbon (Gu et al. 1994; Scheel et al. 2007; Yu et al. 2017). The soil at our study site is alkaline with sandy texture and low buffering capacity (Bai et al. 2010). Interaction with clay minerals by polyvalent cation bridges, especially Ca$^{2+}$ and Mg$^{2+}$ ions, is an important stabilization mechanism for organic C across a wide spectrum of neutral and alkaline soils (Oades 1988; von Lützow et al. 2006; Setia et al. 2014). Our study showed that both N and acid additions led to polyvalent cation depletion and reduced Ca-bound C stocks (Fig. 5c, f; Fig. S2; Fig. 6). Losses of these cations are often accompanied with soil acidification. However, the solubility of most Fe mineral phases increases as pH decreases. The observation of increasing Fe-bound C along with decreasing pH indicated that acidification shifted organic-mineral associations from Ca bridging to Fe-bound C (Fig. 5b, c).

The soils in our study system have a sandy texture with comparatively low reactive Fe and Al content (Table S1) relative to weathered soils with more abundant silt and clay-sized fractions, but this should not affect the direction of the underlying responses of Ca- and Mg-bound, or Fe and Al-associated C pools to N-induced
acidification. In fact, smaller total concentrations of metal-associated C phases may have enabled us to detect treatment effects on these pools over decadal timescales. We expect pH-sensitive organomineral interactions to be quantitatively even more important to total soil C in soils with greater reactive metal stocks. The tradeoff between increased Fe-bound C versus the losses in Ca-bound C and other C-mineral complexes resulting from soil acidification is likely to be broadly applicable among soils, but the absolute magnitudes will depend on the initial pH value, pH buffering capacity, and textural and elemental composition of a given soil.

For example, in acidic soils where Ca-bound C is unimportant, N-induced acidification could potentially increase total mineral-associated C stocks as a consequence of increased Fe solubility and the formation of Fe-C co-precipitates. This presents a plausible hypothesis for interpreting increases in mineral-associated C stocks following N addition to acidic, Fe-rich soils with clay texture (Cusack et al. 2011). Taken together, our results suggest that the loss of an organic C fraction chemically bound with polyvalent cation bridges (such as Ca-organic C associations) caused by N-induced acidification, along with reduced microbial C inputs, may function as major mechanisms by which N inputs reduce heavy fraction C in neutral and alkaline soils.

**Implications for grassland ecosystem C sequestration**

Understanding the mechanisms controlling soil organic C storage in response to N deposition is important for accurately predicting ecosystem C sequestration and/or
potential C losses accompanying increased anthropogenic N inputs. Results from our study showed that although reactive N inputs significantly increased bulk soil organic C and light fraction C content, they significantly reduced the stock of mineral-associated C. We suggest that this resulted from two pathways. First, soil acidification resulting from reactive N inputs may significantly reduce the heavy fraction C formation via suppressing microbial degradation of plant litter, growth of microbial biomass, and thus the production of likely precursors of mineral-associated organic matter. Second, N-induced acidification can enhance losses of the existing heavy fraction C by disrupting mineral-C complexes, particularly those involving Ca\(^{2+}\), a factor which was only partially counteracted by increased Fe-associated C formed under acidic conditions. Together, our results indicate that over the long-term, N-induced acidification may constrain C sequestration in ecosystems where Ca- or Mg-bound C dominates the mineral-bound C. These findings suggest that the impacts of N-induced soil acidification on microbial activity and geochemical properties linked to C stabilization should be included as important factors in Earth system models that predict ecosystem C budgets and ecosystem properties under future N deposition/input scenarios.

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**Figure Captions:**

**Figure 1** Proposed conceptual diagram of contrasting effects of N addition on soil particulate organic C and organo-mineral associations. Black arrows refer to positive effects, red arrows refer to negative effects, and dashed arrows refer to uncertain effects. N addition increases soil N availability and then affects plant growth and soil heterotrophic microbial biomass and decomposition activity. The changes in particulate organic C depend on both production of plant biomass and microbial decomposition. N addition can also cause soil acidification due to increased nitrification and the subsequent leaching of base cations with nitrate (NO$_3^-$), as well as due to biological uptake of ammonium (NH$_4^+$), which releases a proton (H$^+$). The leaching of base cations can promote the release of organic C that adsorbs to mineral surface through polyvalent cation bridging. Nitrogen-induced soil acidification can suppress microbial decomposition and increase solubility of Fe and Al, that in turn limit the transformation of plant litter into organo-mineral associations, and enhance Fe/Al-associated C. Thus, C content in organo-mineral associations depends on the
combined effects of these biotic and abiotic processes.

**Figure 2** Responses of above-ground and below-ground biomass to N enrichment (a, b) and acid addition (c, d).

**Figure 3** Responses of cumulative CO₂ production and microbial biomass C to N enrichment (a, b), acid addition (c, d), and the relationship between these microbial parameters and soil pH for the two experiments (e, f). The slopes and intercepts of the linear regressions between pH and microbial respiration: \(\text{slope}_N = 0.39 \pm 0.06; \text{intercept}_N = -0.96 \pm 0.35; \text{slope}_{acid} = 0.26 \pm 0.08; \text{intercept}_{acid} = 0.34 \pm 0.49\). The slopes and intercepts of the linear regressions between pH and microbial biomass C: \(\text{slope}_N = 211.13 \pm 36.85; \text{intercept}_N = -929.52 \pm 225.57; \text{slope}_{acid} = 188.38 \pm 25.63; \text{intercept}_{acid} = -794.01 \pm 154.82\).

**Figure 4** Responses of light fraction C to N enrichment (a), acid addition (b), and relationships between light fraction C and plant biomass for the two experiments (c, d).

**Figure 5** Responses of heavy fraction C, Fe-bound C, and Ca-bound C to N enrichment (a–c) and acid addition (d–f), and relationships between the different C fractions and pH for the two experiments (g–i). The slopes of the linear regressions between between pH and heavy fraction C: \(\text{slope}_N = 0.97 \pm 0.36; \text{slope}_{acid} = 0.73 \pm 0.25\). The slopes of the linear regressions between pH and Fe-bound C: \(\text{slope}_N = -0.19 \pm 0.04; \text{slope}_{acid} = -0.16 \pm 0.05\). The slopes of the linear regressions between pH and Ca-bound C: \(\text{slope}_N = 0.27 \pm 0.06; \text{slope}_{acid} = 0.23 \pm 0.04\).
Figure 6 Structural equation model (SEM) analysis of the effects of N inputs on soil particulate organic C (POC) and mineral-associated organic C (MAOC) via pathways of soil N availability and soil acidification. Results of the optimal model fitting: Chi-square ($\chi^2$) = 30.637, $P = 0.104$, degree of freedom (df) = 22, comparative fit index (CFI) = 0.952, root square mean error of approximation (RMSEA) = 0.157. Numbers at arrows are standardized path coefficients. Arrow thickness represents the strength of the relationships. Black arrows indicate significant positive relationships and red arrows indicate significant negative relationships ($P < 0.05$). Additionally, $R^2$ values associated with response variables indicate the proportion of variation explained by relationships with other variables. MBC, microbial biomass carbon; Fe-C, iron bound carbon; Ca-C, calcium bound carbon. In this model, POC and MAOC indicate light fraction C and heavy fraction C, respectively.
Figure 1
Figure 2

(a) Above-ground biomass (g m⁻²)
(b) Below-ground biomass (g m⁻²)
(c) Addition rate (mol H⁺ m⁻² yr⁻¹)
(d) Addition rate (g N m⁻² yr⁻¹)

Different letters indicate significant differences at p < 0.05.
Figure 3

(a) Cumulative CO₂ (g kg⁻¹ dry soil)
(b) Microbial biomass C (µg kg⁻¹ dry soil)
(c) N: R² = 0.77, P < 0.01
   A: R² = 0.42, P < 0.01
(d) Addition rate (g N m⁻² yr⁻¹)
(e) Addition rate (mol H⁺ m⁻² yr⁻¹)
(f) Soil pH

[Graphs showing data and significance levels]
Figure 4

(a) Organic C in light fraction (g kg\(^{-1}\) dry soil) vs. Addition rate (g N m\(^{-2}\) yr\(^{-1}\)).

(b) Organic C in light fraction (g kg\(^{-1}\) dry soil) vs. Addition rate (mol H\(^+\) m\(^{-2}\) yr\(^{-1}\)).

(c) Organic C in light fraction (g kg\(^{-1}\) dry soil) vs. Above-ground biomass (g m\(^{-2}\)).

(d) Organic C in light fraction (g kg\(^{-1}\) dry soil) vs. Below-ground biomass (g m\(^{-2}\)).

N: R\(^2\) = 0.51, P < 0.01
A: R\(^2\) = 0.15, P = 0.13

N: R\(^2\) = 0.39, P < 0.01
A: R\(^2\) = 0.06, P = 0.35
Figure 5

(a) Organic C in heavy fraction (g kg⁻¹ dry soil)

(b) Fe-bound C (g kg⁻¹ dry soil)

(c) Ca-bound C (g kg⁻¹ dry soil)

Addition rate (g N m⁻² yr⁻¹)

0, 1.75, 10.5, 25

Addition rate (mol H⁺ m⁻² yr⁻¹)

0, 2.7, 11.04, 16.56

Soil pH

(g) N addition (A: R² = 0.62, P < 0.01)

(h) A: R² = 0.62, P < 0.01

(i) A: R² = 0.65, P < 0.01
Figure 6

Diagram showing the relationship between N inputs, N availability, soil pH, plant biomass, MBC, Fe-C, Ca-C, POC, and MAOC. The diagram includes correlation coefficients and R^2 values for each relationship.