



Nitrogen addition alters elemental stoichiometry within soil aggregates in a temperate steppe

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Abstract. Ongoing increases in anthropogenic nitrogen (N) inputs have largely affected soil carbon (C) and nutrient cycling in most terrestrial ecosystems. Numerous studies have concerned the effects of elevated N inputs on soil dissolved organic carbon (DOC), dissolved inorganic N (DIN), available phosphorus (AP), exchangeable calcium (Ca) and magnesium (Mg), and available iron (Fe) and manganese (Mn). However, few have emphasized the stoichiometric traits of these soil parameters, especially within different soil aggregate fractions. In a semiarid grassland of Inner Mongolia, we studied the effect of N addition on the ratios of DOC : DIN, DOC : AP, DIN : AP, exchangeable Ca : Mg, available Fe : Mn within three soil aggregate classes of large macroaggregates ($> 2000\ \mu\text{m}$), small macroaggregates ($250\text{--}2000\ \mu\text{m}$), and microaggregates ($< 250\ \mu\text{m}$). Elevated N inputs significantly decreased the DOC : DIN ratio within three soil aggregates. The soil DOC : AP ratio significantly decreased along with increasing N gradients within large macroaggregates and microaggregates. Nitrogen significantly decreased the ratio of exchangeable Ca : Mg within soil macroaggregates. The ratio of available Fe : Mn decreased with N addition within three soil aggregate classes. Alteration of elemental stoichiometry within soil fractions that are characterized by different nutrient retention capacity will influence the chemical composition of soil microorganisms and plant quality.

1 Introduction

The use of chemical fertilizers in agriculture is widespread today (Novara et al., 2016; Zupanc et al., 2016). This is resulting in environmental damages to the soil system as chemical fertilizers are part of the modernization of the agriculture around the world and trigger soil degradation and erosion (Cerdà et al., 2009; Ochoa-Cueva et al., 2015; Rodrigo Comino et al., 2016). Grasslands are one of the landscapes that need more attention to the impact of management (Lü et al., 2016; Pereira et al., 2016) as other agriculture landscapes are already well studied (Beniston et al., 2016; de Oliveira et al., 2015; Moreno et al., 2016). The abuse of nitrogen (N) in the agriculture fields is a well-known topic but little is known about its impacts on the grassland soil ecosystems (Liu et al., 2016; Zhang et al., 2016).

Stoichiometric relationships of soil nutrients are reliable indicators of nutrient availabilities for plants and soil microorganisms (Cleveland and Liptzin, 2007). Soil carbon : nitrogen : phosphorus (C : N : P) ratios play essential roles in shaping plant and microbial stoichiometry, which reveals nutrient allocations, life history strategies, and physiological adjustment to climate change (Elser et al., 2010). For instance, enhanced N inputs resulted in higher soil N availability (Wei et al., 2013) and consequentially a decrease in foliar C : N and an increase in the foliar N : P ratio (Han et al., 2014). Nitrogen addition was suggested to cause divergent effects on the plant C : P ratio, which declined at species level but showed no change at the community level (Han et al., 2014). Plant species that are stoichiometric N : P flexibility may have an advantage over those possessing strict stoi-

chiometric homeostasis under environmental changes (Sardans and Peñuelas, 2012). Leaves with high nutrient concentrations (both N and P), which are relatively lower C:N and C:P ratios, tend to be short lived, with a high specific leaf area (Wright et al., 2004) as well as a high photosynthetic capacity and dark respiration rates (Reich et al., 2008). Local nutrient availability from soils is suggested to be one of the most important contributors to the wide variation of terrestrial plants in C:N:P ratios (i.e., plants are what they root in; Elser et al., 2010). Soil exchangeable calcium (Ca) and magnesium (Mg) and available iron (Fe) and manganese (Mn) are critical metal nutrients for plant growth, microbial activity, and ecosystem health (Lucas et al., 2011). The ratio of exchangeable Ca:Mg indicates the relative availability of these two ions and is reported to influence clay dispersion and surface sealing processes (Dontsova and Norton, 2002). Maintaining a properly available Fe:Mn ratio is essential for plant health as a lower ratio might suggest plants suffering from Fe deficiency and Mn toxicity (Hodges, 2010). However, the stoichiometric qualification of soil DOC and available nutrients is less known than plant stoichiometry.

Ongoing increases in anthropogenic N inputs have largely affected soil C and nutrient cycles in most terrestrial ecosystems (Lü et al., 2013; Neff et al., 2002). Under N enrichment, higher soil available N (sum of nitrate and ammonium) has been documented to be positively (Wei et al., 2013), negatively (Heffernan and Fisher, 2012), or even neutrally (Liu and Greaver, 2010) related to dissolved organic C (DOC), which is an important linkage for plant–soil–microbe systems in natural ecosystems. Anthropogenic increases in reactive N would also shift ecosystem nutrient limitation from N to P by stimulating P uptake by plants (Menge and Field, 2007). Moreover, soil acidification caused by N enrichment could help desorption and dissolution of soil inorganic P, serving as an essential P source for plants, especially in calcareous soils (Lajtha and Bloomer, 1988; Tunesi et al., 1999). Soil pH is widely recognized as one of the most influential factors that regulates nutrient bioavailability (Kemmitt et al., 2006). For example, Piccolo (2001) suggested that a drop in soil pH elevated the solubility of soil humic substances. Soil pH is also suggested to influence the soil C:N ratio via changes in plant litter quality (Schmidt, 1982) and increase soil available P (AP) by promoting phosphomonoesterase activity (Hogan et al., 2010). Thus, N enrichment might influence soil available C:N:P stoichiometry through the alteration of soil pH, and hence aggravate or alleviate plant and microbial C and nutrient limitations (Elser et al., 2010). Enhanced N inputs cause leaching of soil exchangeable Ca and Mg and activation of soil available Fe and Mn as a result of soil acidification, which will lead to nutrient imbalance in soils (Katou, 2002; Malhi et al., 1998). For example, N deposition decreases the ratio of exchangeable Ca:Mg as preferential weathering of soil exchangeable Ca relative to Mg (Lu et al., 2014). Iron deficiency chlorosis has been found in calcareous soils in grasslands of America (Rogovska et

al., 2007) and Mn toxicity may occur after soil acidification accompanying leaching of base cations (Lynch and Clair, 2004). Thus, the available Fe:Mn ratio will be useful in indicating the relative availability of Fe and Mn as affected by N addition. Under anthropogenic N enrichment scenarios, clarifying stoichiometric traits of soil available elements would promote our understanding of soil nutrient imbalance as caused by environmental changes.

Physical protection by soil aggregation is a main mechanism of soil organic matter (SOM) stabilization (Amezketta, 1999; Wiesmeier et al., 2012). Oxygen and water diffusion rates are generally parallel with soil aggregate sizes as determined by pore space (Young and Ritz, 2000; Baker et al., 2007). Consequently, available nutrients like N and P tend to distribute in aggregates of larger size due to more favorable microclimates for microbial activity herein (Dorodnikov et al., 2009; Wu et al., 2012). Smaller aggregate-size classes are confirmed as a preferential site for the physical stabilization of SOM and nutrient retention (Fonte et al., 2014). Higher clay contents in microaggregates tend to provide more surface area and binding sites to retain more exchangeable Ca and Mg (Oorts et al., 2003). Within microaggregates, more microbial-processed SOM will also chelate or make complex a larger amount of base cations and micronutrients (Lü et al., 2016). Even though numerous studies have focused on C and nutrient cycling in the aggregate scale (Fonte et al., 2014; Six and Paustian, 2014; Wright, 2009), stoichiometric traits of DOC and available nutrients within aggregates are still unclear.

Semiarid steppes of Inner Mongolia in northern China, playing a vital role in serving environmental health, regional economy, and the global C cycle, have undergone degradation during the last 50 years' increased stocking rates and static grazing management practices (Kang et al., 2007). Non-physically protected SOM pools, which can be a majority of SOC and nutrients in these sandy grassland systems, are, however, inherently vulnerable to environmental changes (Creamer et al., 2011), like elevated N inputs. A 7-year N field manipulation experiment has already shown significant aboveground changes in species turnover rates, plant community composition, and community stability (Xu et al., 2012). Microaggregates (<250 µm) had the highest dissolved inorganic N (DIN) concentration as compared to large (>2000 µm) and small (250–2000 µm; see Wang et al., 2015a) macroaggregates, which was not the case for both DOC (see Wang et al., 2015b) and AP (Wang et al., 2016a). Nitrogen addition significantly increased DIN (see Wang et al., 2015a) and available Fe and Mn but decreased exchangeable Ca and Mg across three soil fractions (not for Ca in microaggregates; Wang et al., 2016b). In this study, we aimed to analyze stoichiometric traits of DOC, DIN, AP, exchangeable Ca and Mg, and available Fe and Mn within soil aggregates in this semiarid grassland. We hypothesize that microaggregates had lower DOC:DIN and higher DIN:AP ratios due to better N retention capacity in smaller-size aggregates. We

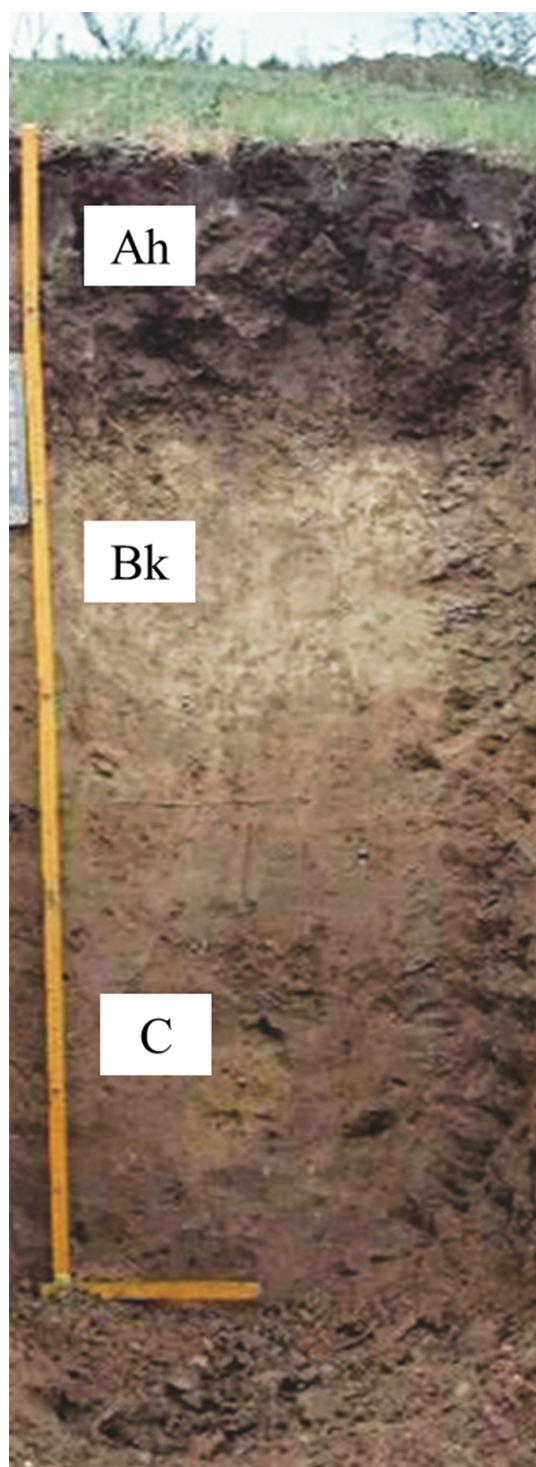


Figure 1. Schematic diagram of chestnut soil illustrating three soil horizons (figure modified from the Digital Science and Technology Museum of China, http://amuseum.cdstm.cn/AMuseum/agricul/6_5_27_ligt.html).

also hypothesized that N addition would decrease DOC : DIN while increasing DOC : AP and DIN : AP ratios due to higher anthropogenic inputs of inorganic N and intensive removal of available P from soil by plant uptake. Due to preferential weathering of Ca relative to Mg during soil acidification, we expected to detect a significant decrease in the Ca : Mg ratio under N addition across soil aggregate classes. Due to the fact that plant growth is commonly limited by Fe deficiency in calcareous grasslands, enhanced Fe translocation from soil to plants would result in a lower soil available Fe : Mn ratio under N enrichment.

2 Methods

2.1 Study site and experimental design

The study site ($42^{\circ}02'27''$ N, $116^{\circ}17'59''$ E, elevation 1324 m a.s.l.) was located in Duolun County, a semiarid area in Inner Mongolia, China. The mean annual temperature is 2.1° C, ranging from 17.8° C in January to 18.8° C in July, and average precipitation is 379.4 mm yr^{-1} . The soil is chestnut in the Chinese classification and Calcisorthic Aridisol in the US Soil Taxonomy classification. The soil texture of the experimental site is sandy loam with 62.75 % sand, 20.30 % silt, and 16.95 % clay (Liu et al., 2009). The soil contains an Ah horizon of 25–50 cm thickness with dark brown color, a Bk horizon of 30–50 cm thickness with grayish color, and a C horizon with dark-grayish brown color (Fig. 1; Heil and Buntley, 1965; Buol et al., 2011).

In April 2005, seven blocks ($107 \text{ m} \times 8 \text{ m}$) were set up in a split-plot design with water and N being the two treatments. Each block was divided into two main plots receiving either ambient precipitation or additional water treatment (180 mm). Each main plot was divided into six sub-plots which were randomly treated with four levels of N (0 [CK], 5 [N₅], 10 [N₁₀], and 15 [N₁₅] $\text{g N m}^{-2} \text{ yr}^{-1}$), P (10 $\text{g N m}^{-2} \text{ yr}^{-1}$), and combined N and P addition. This study was only concerned about the treatments of four levels of N addition under ambient precipitation. Nitrogen was added as urea pellets, with half of it applied in early May and the other half in late June.

2.2 Field sampling and measurements

Soil samples (0–10 cm) were collected from each N plot from four out of seven blocks in August 2013. For each plot, five random cores were retrieved after removing the litter layer and stored at 4° C under further analysis. Soil aggregates were isolated according to Dorodnikov et al. (2009) into three aggregates ($>2000 \mu\text{m}$, large macroaggregate class; $250\text{--}2000 \mu\text{m}$, small macroaggregates; and $<250 \mu\text{m}$, microaggregate class) by a Retsch AS 200 Control (Retsch Technology, Düsseldorf, Germany). This dry-sieving method was used to maintain microbial activity and reduce the loss of DOC and available nutrients in soil aggregates. The sepa-

Table 1. The concentrations of soil organic carbon (SOC, g kg soil aggregate⁻¹), total nitrogen (g kg soil aggregate⁻¹), dissolved organic carbon (DOC, mg kg soil aggregate⁻¹), dissolved inorganic nitrogen (DIN, mg kg soil aggregate⁻¹), available phosphorus (AP, mg kg soil aggregate⁻¹), exchangeable Ca and Mg (cmol kg soil aggregate⁻¹), and available Fe and Mn (mg kg soil aggregate⁻¹) within bulk soil and soil fractions of 0–10 cm soils without field-manipulated treatments (data from Wang et al., 2015a, b, and 2016a).

	Soil aggregates (μm)			
	Bulk soil	> 2000	250–2000	< 250
SOC	18.9 ± 1.7	18.2 ± 1.0	15.1 ± 2.0	21.6 ± 1.4
TN	1.8 ± 0.2	1.9 ± 0.1	1.5 ± 0.2	2.2 ± 0.1
DOC	69.8 ± 5.6	72.5 ± 6.8	66.6 ± 1.3	71.2 ± 1.5
DIN	21.6 ± 1.5	21.1 ± 0.6	49.5 ± 1.9	57.3 ± 2.0
AP	14.9 ± 2.8	3.7 ± 0.4	5.8 ± 0.7	3.9 ± 0.5
Ca	19.6 ± 0.3	26.6 ± 0.7	19.8 ± 0.4	22.7 ± 2.7
Mg	1.7 ± 0.05	1.8 ± 0.1	1.4 ± 0.05	2.0 ± 0.1
Fe	21.8 ± 0.6	24.0 ± 1.8	20.0 ± 1.4	27.5 ± 2.8
Mn	23.3 ± 2.7	24.5 ± 0.8	20.0 ± 0.8	24.0 ± 1.9

rated aggregates were weighed and used for determining soil aggregate moisture (drying at 105°C for 48 h), DOC, DIN, AP, exchangeable Ca and Mg, and available Fe and Mn.

Soil pH was measured in a 1 : 2.5 (*w/v*) soil-to-water slurry of soil aggregate samples with a digital pH meter. The concentration of DOC was extracted by adding 50 mL of 0.5 M potassium sulfate and analyzed by a TOC analyzer (High TOC, Elementar). Soil DIN concentration was extracted with 50 mL 2 M KCl from 10 g fresh soils (McLeod, 1992) and determined colorimetrically using an AutoAnalyser III continuous Flow Analyzer (Bran & Luebbe, Norderstedt, Germany). Soil AP was measured using the Olsen method (Olsen et al., 1954). Briefly, 2.5 g of the soil fraction was mixed with 50 mL 0.5 M NaHCO₃ (pH 8.5) and 5 g of phosphorus-free charcoal. The mixture was shaken at 150 rpm for 30 min and filtered. The P concentration of P in filtration was determined by the molybdenum blue colorimetric method (Murphy and Riley, 1962). The units of DOC, DIN, and AP were expressed in mg per kg soil aggregate. Exchangeable Ca and Mg within soil aggregates were extracted by mixing a 2.5 g soil fraction with 50 mL 1 M ammonium acetate (pH 7.0) and then shaken for 30 min at 150 rpm. After filtration, the Ca and Mg concentrations in extraction were determined by an atomic absorption spectrometer (AAS, Shimadzu, Japan). To analyze available Fe and Mn within soil aggregate fractions, 10 g of soil samples were extracted by 20 mL 0.005 M DTPA + 0.01 M CaCl₂ + 0.1 M TEA (triethanolamine; pH 7.0). After being shaken at 180 rpm for 2 h, the extracts were filtered and available Fe and Mn concentrations in the filtration were determined by the AAS. Soil DOC and available nutrients of the control plot were listed in Table 1 (data from Wang et al., 2015a, b, and 2016a). The

Table 2. Two-way ANOVAs (*F* values) of the effect of soil aggregate size (S), nitrogen addition (N), and their interactions on the ratios of DOC : DIN, DOC : AP, DIN : AP, exchangeable Ca : Mg, and available Fe : Mn.

	DOC : DIN	DOC : AP	DIN : AP	Ca : Mg	Fe : Mn
S	100.86**	0.41	16.32**	1.81	0.25
N	36.91**	3.44*	1.73	1.19	109.23**
N × S	12.64**	1.72	2.43*	2.07	7.41**

* Significance level at *P* < 0.05. ** Significance level at *P* < 0.01.

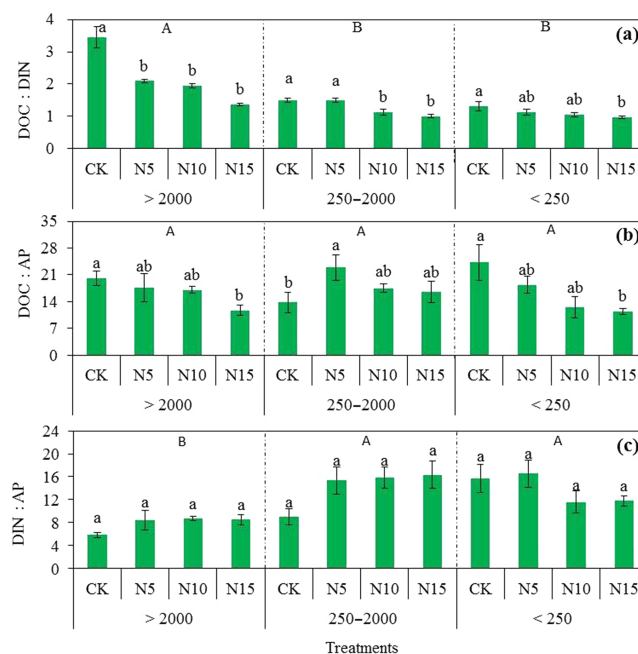


Figure 2. The ratio of (a) dissolved organic carbon to dissolved inorganic nitrogen (DOC : DIN), (b) DOC to available phosphorus (DOC : AP), and (c) DIN : AP as influenced by N addition (0 [CK], 5 [N₅], 10 [N₁₀], and 15 [N₁₅] g N m⁻² yr⁻¹) in soil aggregates. Data are represented as mean ± SE. Lowercase letters indicate significant differences between N treatments within a soil fraction. The capital letters at the top indicate significant differences between soil aggregate sizes across N treatments.

concentrations of exchangeable Ca and Mg were represented as cmol per kg soil aggregate, and it was a mg per kg soil aggregate for available Fe and Mn.

2.3 Statistical analysis

The normality of data and homogeneity of variances were determined by the Kolmogorov–Smirnov test and Levene's test, respectively. Two-way ANOVAs were executed to determine soil aggregate size (S), N addition, and their interactive effects (S × N) on the ratios of DOC : DIN, DOC : AP, DIN : AP, exchangeable Ca : Mg, and available Fe : Mn. Multiple comparisons with a Duncan design were conducted to determine the difference between N treatments within each

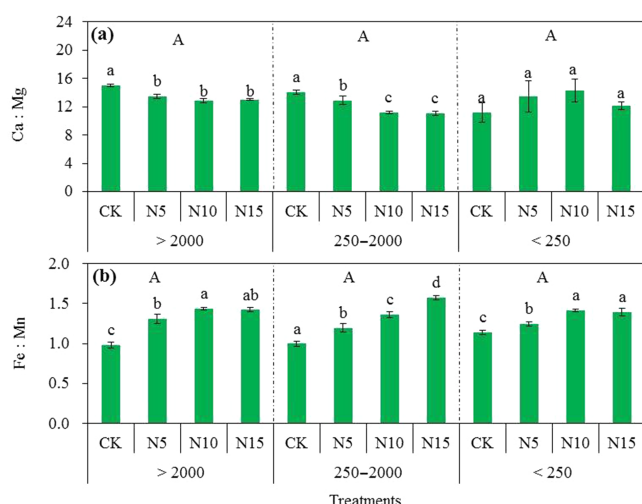


Figure 3. The ratio of (a) exchangeable Ca : Mg and (b) available Fe : Mn as influenced by N addition (0 [CK], 5 [N₅], 10 [N₁₀], and 15 [N₁₅] g N m^{−2} yr^{−1}) in soil aggregates. Data are represented as mean ± SE. Lowercase letters indicate significant differences between N treatments within a soil fraction. The capital letters at the top indicate significant differences between soil aggregate sizes across N treatments.

soil fraction. Pearson correlation analysis was used to determine the relationship between soil pH and these ratios within each soil aggregate class. The whole correlation analysis was performed in SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and statistical significance was accepted at $P < 0.05$.

3 Results

3.1 N addition effects on soil available C : N : P

The ratio of DOC : DIN varied from 1.4 to 3.4 within large macroaggregates, from 1.0 to 1.5 within small macroaggregates, and from 1.0 to 1.3 within microaggregates (Fig. 2a). The DOC : DIN ratio was significantly higher in large macroaggregates than in both small macroaggregates and microaggregates, averaging across all N treatments (Fig. 2a). Compared to CK, the DOC : DIN ratio was significantly lower in N₅, N₁₀, and N₁₅ treatments of large macroaggregates, in N₁₀ and N₁₅ of small macroaggregates, and in N₁₅ of microaggregates (Fig. 2a). The DOC : DIN ratio significantly decreased under N₅ and N₁₀ treatments in large macroaggregates, but not for N₅ in small macroaggregates and N₅ and N₁₀ in microaggregates, resulting in a significant interaction between N and aggregate size (S; $N \times S$; Table 2).

No difference in the soil DOC : AP ratio was found among three aggregate classes (Fig. 2b). The soil DOC : AP ratio significantly decreased under N₁₅ treatment in both large macroaggregates and microaggregates, but it increased under N₅ in small macroaggregates (Fig. 2b). No significant interactive $N \times S$ was detected in the DOC : AP ratio (Table 2).

The DIN : AP ratio was significantly lower in large macroaggregates as compared to both small macroaggregates and microaggregates (Fig. 2c). The soil DIN : AP ratio showed no change along N gradients as compared to CK within three soil aggregates (Fig. 2c). There was a significant interaction of $N \times S$ in the DIN : AP ratio (Table 2).

3.2 Nitrogen addition effects on exchangeable Ca : Mg and available Fe : Mn ratios

The exchangeable Ca : Mg ratio ranged from 11.0 to 15.0 across soil aggregate classes (Fig. 3a). Nitrogen addition significantly decreased the Ca : Mg ratio compared to CK within large and small macroaggregates (Fig. 3a). Neither aggregate size nor interactive N and aggregate size effects were detected in changes in the Ca : Mg ratio.

The ratio of available Fe : Mn ranged from 0.98 to 1.57 across three soil aggregate fractions (Fig. 3b). No difference in the Fe : Mn ratio was detected among three soil fractions (Fig. 3b, Table 2). Nitrogen addition significantly increased soil available Fe : Mn within three soil fractions (Fig. 3b). Nitrogen addition significantly interacted with soil aggregate size to affect the ratio of available Fe : Mn (Table 2).

3.3 Correlation analyses

The DOC : DIN ratio was positively correlated with soil pH within three soil fractions (Fig. 4a, b, c). A significantly positive relationship was found between the DOC : AP ratio and soil pH within large macroaggregates and microaggregates (Fig. 4d, f). A significant and negative correlation between the DIN : AP ratio and soil pH was only detected in small macroaggregates (Fig. 4h). Within large and small macroaggregates, the Ca : Mg ratio was significantly and positively correlated with soil pH (Fig. 5a, b). Significant and negative relationships between the Fe : Mn ratio and soil pH were found within three soil fractions (Fig. 5d, e, f).

4 Discussion

4.1 The stoichiometric ratios among different soil aggregates fractions

Consistent with our initial hypothesis, microaggregates had lower DOC : DIN ratios and higher DIN : AP ratios relative to large macroaggregates. It is generally recognized that more labile SOM concentrates in macroaggregates, with more recalcitrant SOM in microaggregates (Jastrow et al., 2007). Additionally, SOM in microaggregates experiences more microbial processing cycles which are more decomposed as compared to macroaggregates (Six and Paustian, 2014). These resulted in a lower C : N of SOM in microaggregates. Our previous study suggested that microaggregates retained significantly higher inorganic N relative to large macroaggregates (Wang et al., 2015a). However, DOC and AP con-

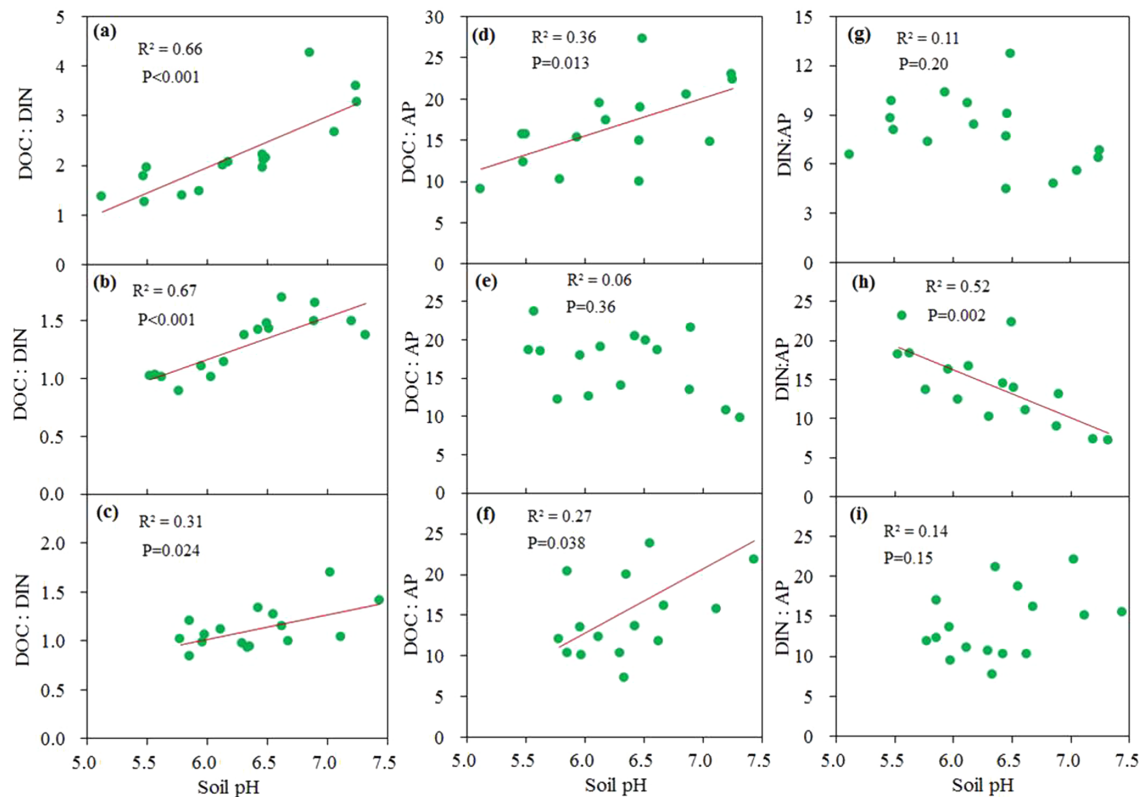


Figure 4. The relationships between soil pH and DOC : DIN within large macroaggregates (a), small macroaggregates (b), and microaggregates (c), between soil pH and DOC : AP within large macroaggregates (d), small macroaggregates (e), and microaggregates (f), and between soil pH and the DIN : AP ratio within large macroaggregates (g), small macroaggregates (h), and microaggregates (i). The significance level was set at $P < 0.05$.

centrations showed no difference between large macroaggregates and microaggregates, as suggested by our previous studies (Wang et al., 2015b, 2016a). In this context, microaggregates had significantly lower DOC : DIN ratios (Fig. 2a) but higher DIN : AP ratios (Fig. 2c) than large macroaggregates. Our results indicated that microaggregates were preferential sites for retaining DIN relative to DOC and AP as compared to large macroaggregates (Fig. 2a,c). Microaggregates serve as an indicator of soil C storage capacity and help elucidate the capacity of soils to protect SOM and supply N and P nutrients (Du et al., 2015). Higher DIN : AP ratios in microaggregates would be essential for available N supply, but it might eventually result in P deficiency under enhanced N loading rates in the long term.

4.2 Effect of N addition on DOC : DIN, DOC : AP, and DIN : AP ratios within soil aggregates

As expected, we observed a significant decrease in the soil DOC : DIN ratio within all soil aggregate classes affected by N addition (Fig. 2a). This should mainly be due to a supply of DIN which is directly transformed from urea fertilizer or indirectly derived from enhanced soil organic N mineralization and nitrification under urea addition (Chen et al., 2013)

within three soil fractions. Indeed, our previous studies suggested that N addition increased DIN by up to 128.3, 41.2, and 45.7 % (Wang et al., 2015a), while DOC only increased by 12.7, 3.7, and 12.7 % within large macroaggregates, small macroaggregates, and microaggregates, respectively (Wang et al., 2015b). As suggested by our previous work, N addition decreased soil pH from 7.3 to 5.6 in large macroaggregates, from 7.5 to 5.8 in small macroaggregates, and from 7.4 to 5.9 in microaggregates (Wang et al., 2015b). Soil pH was one of the main factors influencing the DOC : DIN ratio, as suggested by their positive correlations within three soil fractions (Fig. 4a, b, c). A decline in soil pH was reported to be associated with accumulation of soil DIN (Aciego Pietri and Brooks, 2008), thus leading to the decrease in the DOC : DIN ratio (Fig. 3a, b, c).

Soil DOC and DIN are important energy and N sources for microorganisms, and relative soil C and N availabilities (represented as DOC : DIN) make a great contribution to controlling microbial activity and nutrient cycling processes (Dijkstra et al., 2006; Wu et al., 2012). A significant decrease in DOC : DIN (Fig. 1a) might suggest that higher N inputs induced microbial C limitation relative to N across soil fractions. However, Wei et al. (2013) found that N ad-

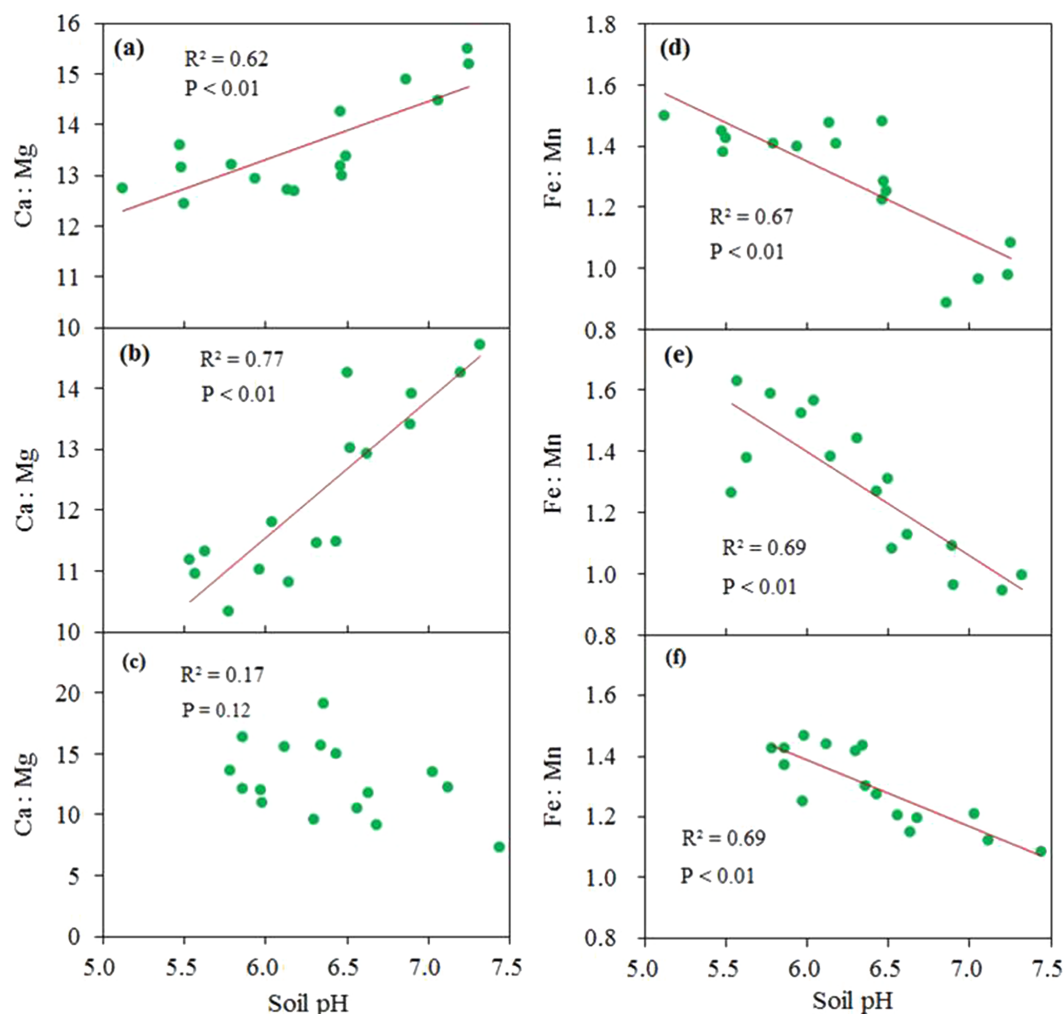


Figure 5. The relationships between soil pH and exchangeable Ca : Mg within large macroaggregates (a), small macroaggregates (b), and microaggregates (c), and between soil pH and the available Fe : Mn ratio within large macroaggregates (d), small macroaggregates (e), and microaggregates (f). The significance level was set at $P < 0.05$.

dition increased both DOC and DIN but decreased microbial biomass C (MBC) concentration, and they suggested that soil microbes were no longer C-limited due to a non-significant correlation between DOC and microbial biomass C. In their study, they failed to analyze the relationship between the DOC : DIN ratio and MBC, which could be used to indicate relative C and N availabilities in determining microbial growth. In this study, a coincident decrease in the DOC : DIN ratio (Fig. 2a) and MBC (see Wang et al., 2015b) indicated that repression of microbial growth under N addition was due to lower C availability relative to N (Treseder, 2008).

Our previous study suggested that N addition increased soil AP by 60.2 and 84.5 % within large macroaggregates and microaggregates, respectively (Wang et al., 2016a). Based on this, it was reasonable to detect a significant decrease in the ratio of DOC : AP within large macroaggregates and microaggregates (Fig. 2b). Soil acidification could enhance dis-

solution of phosphate from mineral-bound P pools, while this was not the case for DOC (Wang et al., 2016a). Thus, production of DOC might not keep up with continuous dissolution of AP, resulting in a lower DOC : AP ratio under N addition (Fig. 2b). Significant positive correlations between soil pH and the DOC : AP ratio (Fig. 4d, f) also confirmed higher AP dissolution than DOC supply under soil acidification. Under the scenario of enhanced ecosystem N inputs, a decrease in the soil aggregate DOC : AP ratio would induce microbial C limitation relative to P (Treseder, 2008), and continuous release of AP from rock weathering would shift the ecosystem from N limitation to P limitation in the long term (Lü et al., 2013).

4.3 Effect of N addition on exchangeable Ca : Mg and available Fe : Mn ratios

Within large and small macroaggregates, a significant decrease in the exchangeable Ca : Mg ratio supported our hypothesis. Soil acidification, as developed under increased SO_2 and NO_x emissions, results in weathering and release of base cations (Lucas et al., 2011). Selective weathering of certain base cations ($\text{Ca} > \text{Na} > \text{Mg} > \text{K}$) would result in a change in base cation budgets and an imbalance of metal ions in soils (Lu et al., 2014). Preferential loss of exchangeable Ca relative to Mg was the main reason for the significant decrease in the Ca : Mg ratio in large and small macroaggregates (Fig. 3a). Correlation analyses also suggested a coincident decline in the Ca : Mg ratio and soil pH, indicating that more exchangeable Ca was leached than Mg during soil acidification in macroaggregates. However, the unaffected Ca : Mg ratio in microaggregates contradicted our hypothesis that the Ca : Mg ratio would decrease across three soil fractions. In this sandy grassland, microaggregates containing more minerals and less sand would be a more favorable site for base cation binding and adsorption, which was less affected by N addition and sequential soil acidification compared to macroaggregates (Gunina and Kuzyakov, 2014). Moreover, more decomposed SOM as indicated by $\delta^{13}\text{C}$ (Wang et al., 2015a) would provide an additional strong binding surface for exchangeable Ca and Mg in microaggregates. Thus, a significant relationship of the Ca : Mg ratio with soil pH was not detected in microaggregates (Fig. 5c).

The soil Ca : Mg ratio affects plant Cu uptake and lower Ca : Mg might increase or decrease plant Cu uptake and toxicity depending on plant species and soil types (Lombini et al., 2003). Under enhanced N inputs, a significant decrease in the Ca : Mg ratio would further influence nutrient balance, i.e., Cu uptake of plants in this calcareous grassland. Researchers proposed that relative availabilities of exchangeable Ca and Mg were more important than their absolute amounts (Bear et al., 1951; Albrecht, 1975; Kopittke and Menzies, 2007), and optimal Ca : Mg ratio ranges for plant growth were identified for various plant species since the study from Loew (1892). In addition, different soil aggregate classes were characterized by their various nutrient supply capacities (Mikha and Rice, 2004). Soil structure could modify the effect of cation ratios on plant growth (Zhang and Norton, 2002). These shed light on the essential role of studying the soil exchangeable Ca : Mg ratio of different soil aggregate fractions in evaluating soil fertility and plant growth.

For all soil fractions, a significant increase in the available Fe : Mn ratio contradicted our initial hypothesis. A decrease in the Fe : Mn ratio might be caused by the antagonistic relationship between Fe and Mn during plant uptake (Somers and Shive, 1942). Soil acidification promoted weathering and desorption of micronutrients from soil minerals, which increased soil available Fe and Mn concentrations (Malhi et al., 1998). Even though Fe availability generally limits pro-

ductivity in calcareous grasslands, enhanced plant Mn uptake might retard plant Fe acquisition, leading to higher available Fe retained in soil (Tanaka and Navasero, 1966; Tian et al., 2016) under soil acidification. Correspondingly, negative relationships of soil pH with Fe : Mn ratios (Fig. 5d, e, f) suggested accumulation of available Fe relative to Mn under soil acidification as affected by enhanced N inputs. Consistent with our findings, Tian et al. (2015, 2016) found soil acidification enhanced Mn uptake and reduced Fe absorption by forbs which result in lower photosynthetic rates and growth and loss of forb species. Plant Fe deficiency was reported to induce chlorosis and decrease photosynthesis in calcareous grassland (Rogovska et al., 2007). An increase in the soil available Fe : Mn ratio might pose a threat to grassland productivity such as potential aggravation of Fe deficiency and the occurrence of Mn toxicity to plant species (Tian et al., 2016). Soil nutrient imbalance would not only influence both the quantity (yield) and quality of plants, but also cause an impact on the health of animals grazing on these plants (Mikha and Rice, 2004).

5 Conclusions

After 9-year N amendment, we found that microaggregates had lower DOC : DIN but higher DIN : AP ratios. The soil DOC : DIN ratio decreased with N addition within three fractions as a result of exogenous N supply. Nitrogen addition decreased the DOC : AP ratio in large macroaggregates and microaggregates which might induce microbial C limitation relative to P. Nitrogen addition significantly decreased the exchangeable Ca : Mg ratio within macroaggregates purportedly due to preferential leaching of exchangeable Ca under soil acidification as developed with N addition. Within three soil aggregate classes, a significant increase in the available Fe : Mn ratio might cause plant Fe deficiency and Mn toxicity as affected by ecosystem N enrichment. Changes in the ratios of available elements among soil fractions that are responsible for different nutrient retention and cycling processes will cause nutrient imbalance, affect plant and microbial nutrient acquisition and chemical composition, and influence ecosystem productivity and health.

6 Data availability

Data are available at <https://datadryad.org/resource/doi:10.5061/dryad.333f1>.

Author contributions. Yong Jiang and Zhuwen Xu designed the experiment; Jinfei Yin, Heyong Liu, and Ruzhen Wang carried them out. Xue Feng helped to do the laboratory analyses. Ruzhen Wang prepared the manuscript with contributions from all authors.

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