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

Jinfei Yin [1,2], Ruzhen Wang [1,*], Heyong Liu [1], Xue Feng [1], Zhuwen Xu [1], Yong Jiang [1]

[1] State Engineering Laboratory of Soil Nutrient Management, Institute of Applied

Ecology, Chinese Academy of Sciences, Shenyang 110016, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

Correspondence to: Ruzhen Wang (ruzhenwang@iae.ac.cn)

Abstract

Ongoing increases in anthropogenic nitrogen (N) inputs have largely affected soil carbon (C) and nutrients 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, little has emphasized on stoichiometric traits of these soil parameters, especially within different soil aggregate fractions. In a semi-arid grassland of Inner Mongolia, we studied 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 μm), small macroaggregates (250-2000 μm), and microaggregates (< 250 μm). Elevated N inputs significantly decreased DOC:DIN ratio within three soil aggregates. Soil DOC:AP ratio significantly decreased along with increasing N gradients within large macro- 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 featured by different nutrient retention capacity will influence chemical composition of soil microorganisms and plant quality.

Key words: nitrogen deposition, soil fraction, C:N:P stoichiometry, Ca:Mg ratio, Fe:Mn ratio

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 decrease of foliar C:N and increase of foliar N:P ratio (Han et al., 2014). Nitrogen addition was suggested to cause divergent effects on 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 advantage over these possessing strict stoichiometric homeostasis under environmental changes

(Sardans and Peñuelas, 2012). Leaves with high nutrient concentrations (both N and P), which is relatively lower C:N and C:P ratios tend to be short lived with high specific leaf area (Wright et al., 2004) as well as high photosynthetic capacity and dark respiration rates (Reich et al., 2008). Local nutrient availability from soils is

5   suggested to be one of the most important contributors to the wide variation of terrestrial plants in C:N:P ratios (i.e. plant 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

10 relative availability of these two ions and is reported to influence clay dispersion and surface sealing processes (Dontsova and Norton, 2002). Maintaining proper available Fe:Mn ratio is essential for plant health as 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

15 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,

20 2012) or even neutrally (Liu and Greaver, 2010) related to dissolved organic C (DOC), which is an important linkage for plant-soil-microbe system 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

5   one of the most influential factors that regulates nutrient bioavailability (Kemmitt et al., 2006). For example, Piccolo (2001) suggested that drop of soil pH elevated solubility of soil humic substances. Soil pH is also suggested to influence soil C:N ratio via changes in plant litter quality (Schmidt, 1982) and increase soil available P (AP) through promoting phosphomonoesterase activity (Hogan et al., 2010). Thus, N

10   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 causes 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.,

15   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 grassland of America (Rogovska et al., 2007) and Mn toxicity may occur after soil acidification companying with leaching of base cations (Lynch and Clair, 2004). Thus, available

20   Fe:Mn ratio will be useful in indicating 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 (Amezketa, 1999; Wiesmeier, 2012). Oxygen and water diffusion rates is generally parallel with soil aggregate sizes as being 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 as more favorable microclimate 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 complex larger amount of base cations and micronutrients (Lü et al., 2016). Even though numerous studies have focused on C and nutrients cycling in 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 showed

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 macroaggregates (250-2000 μm) (see Wang et al., 2015a) which

was not the case for both DOC (see Wang et al., 2015b) and AP (Wang et al., 2016).

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., ~~2017~~in press). In this study, ~~W~~we aimed to

analyze~~d~~ stoichiometric traits of DOC, DIN, AP, exchangeable Ca and Mg, and

available Fe and Mn within soil aggregates in this semi-arid grassland. We

hypothesize that microaggregates had lower DOC:DIN and higher DIN:AP ratios as

better N retention capacity in smaller-size aggregates. We also hypothesized that N

addition would decrease DOC:DIN while increase DOC:AP and DIN:AP ratios as

higher anthropogenic inputs of inorganic N and intensive removal of available P from

soil by plant uptake. As preferential weathering of Ca relative Mg during soil

acidification, we expected to detect significant decrease of 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 lower soil available Fe:Mn ratio under N enrichment.

Methods

Study site and experimental design

The study site (42°02′27″N, 116°17′59″E, elevation 1,324 m a.s.l) was located in Duolun County, a semi-arid area in Inner Mongolia, China. Mean annual temperature is 2.1 ℃, ranging from 17.8 ℃ in January to 18.8 ℃ in July and average precipitation is 379.4 mm yr^{-1}. The soil is a chestnut in Chinese classification and Calcisorthic Aridisol in the U.S. Soil Taxonomy classification. 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 Ah horizon of 25-50 cm thickness with dark brown color, Bk horizon of 30-50 cm thickness with grayish color, and C horizon with dark-grayish brown color (Fig. 1) (Heil and Buntley, 1965; Buol et al., 2011).

In April 2005, seven blocks (107 m $\times$ 8 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 subplots which were randomly treated with four levels of N (0 [CK], 5 [N$_5$], 10 [N$_{10}$], and 15 [N$_{15}$] g N m^{-2} yr^{-1}), P (10 g N m^{-2} yr^{-1}), and combined N and P addition. This study 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.

Field sampling and measurements

Soil Samples (0-10 cm) were collected from each N plot from four out of seven blocks on August 2013. For each plot, five random cores were retrieved after removing litter layer and stored at 4 ℃ under further analysis. Soil aggregates were

isolated according to Dorodnikov et al. (2009) into three aggregates (> 2mm, large

macroaggregates class; 2-0.25 mm, small macroaggregates; and < 0.25mm,

microaggregates class) by a Retsch AS 200 Control (Retsch Technology, Düsseldorf,

Germany). This dry-sieving method was used to maintain microbial activity and

5 reduce the loss of DOC and available nutrients in soil aggregates. The separated

aggregates were weighed and used for determining soil aggregate moisture (drying at

105 ℃ 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

10 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

15 fraction was mixed with 50 mL 0.5 M $NaHCO_3$ (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 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

20 extracted by mixing 2.5 g soil fraction with 50 mL 1M 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 atomic absorption spectrometer (AAS, Shimadzu,

Japan). To analyze available Fe and Mn within soil aggregate fractions, 10 g of soil

samples was extracted by 20 mL 0.005 M DTPA + 0.01 M $CaCl_2$ + 0.1 M TEA

(triethanolamine) (pH 7.0). After 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.

5   Soil DOC and available nutrients of control plot was listed in Table 1 (data from

Wang et al. (2015a,b and 2016)). The concentrations of exchangeable Ca and Mg

were represented as cmol per kg soil aggregate and it was mg per kg soil aggregate for

available Fe and Mn.

Statistical analysis

10   The normality of data and homogeneity of variances were determined by

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 was conducted to

15   determine difference between N treatments within each soil fraction. Pearson

correlation analysis was used to determine the relationship between soil pH and these

ratios within each soil aggregate classes. All the correlation analysis was performed in

SPSS 16.0 (SPSS, Inc., Chicago, IL, U.S.A.) and statistical significance was accepted

at P<0.05.

Results

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 macro- and microaggregates averaging across all N treatments (Fig. 2a).

5 ~~Nitrogen addition significantly decreased DOC:DIN ratio within three soil aggregate classes~~Compared to CK, the DOC:DIN ratio was significantly lower in N_5, N_{10}, and N_{15} treatments of large macroaggregates, in N_{10} and N_{15} of small macroaggregate, and in N_{15} of microaggregates (Fig. 2a). The DOC:DIN ratio ~~Significant~~ significantly decreased under N_5 and N_{10} treatments in large macroaggregates but not for N_5 in

10 small macroaggregates and N_5 and N_{10} in microaggregates resulting in a significant interaction between N and aggregate size (S) (N×S) ~~was detected on DOC:DIN ratio~~ (Table 2).

No difference in soil DOC:AP ratio was found among three aggregate classes (Fig. 2b). Soil DOC:AP ratio significantly decreased ~~with~~ under N_{15} ~~addition~~ treatment in

15 both large macro- and microaggregates, but it increased under N_5 in small macroaggregates (Fig. 2b). No significant interactive N×S was detected on DOC:AP ratio (Table 2).

The DIN:AP ratio was significantly lower in large macroaggregates as compared to both small macro- and microaggregates (Fig. 2c). Soil DIN:AP ratio showed no

20 change along N gradients as compared to CK within three soil aggregates (Fig. 2c). There was a significant interaction of N×S on the DIN:AP ratio (Table 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 Ca:Mg ratio comparing to CK within large and small macroaggregates (Fig. 3a). Neither aggregate size nor interactive N and aggregate size effects was detected on changes of 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 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).

Correlation analyses

The DOC:DIN ratio was positively correlated with soil pH within three soil fractions (Fig. 4a,b,c). Significantly positive relationship was found between DOC:AP ratio and soil pH within large macro- and microaggregates (Fig. 4d,f). Significant and negative correlation between 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).

Discussion

The stoichiometric ratios among different soil aggregates fractions

Consistent with our initial hypothesis, microaggregates had lower DOC:DIN ratio

and higher DIN:AP ratio relative to large macroaggregates. It's generally recognized that more labile SOM concentrates in macroaggregates while more recalcitrant SOM in microaggregates (Jastrow et al., 2007). Additionally, SOM in microaggregates experiences more microbial processing cycles which is more decomposed as

5   compared to macroaggregates (Six and Paustian, 2014). These resulted in 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 concentrations showed no difference between large macro- and microaggregates as suggested by our previous studies (Wang et al.,

10   2015b, 2016). In this context, microaggregates had significantly lower DOC:DIN ratio (Fig. 2a) but higher DIN:AP ratio (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

15   capacity of soils to protect SOM and supply N and P nutrients (Du et al., ~~2014~~2015). Higher DIN:AP ratio 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.

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

20   *aggregates*

As expected, we observed a significant decrease of soil DOC:DIN ratio within all soil aggregate classes as affected by N addition (Fig. 2a). This should mainly be due

to 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

5   et al., 2015a), while DOC only by 12.7%, 3.7%, and 12.7% within large macro-, small

macro-, 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 DOC:DIN ratio

10   as suggested by their positive correlations within three soil fraction (Fig. 4a,b,c).

Decline of soil pH was reported to be associated with accumulation of soil DIN

(Aciego Pietri and Brooks, 2008), thus leading to the decrease of DOC:DIN ratio (Fig.

3a,b,c).

Soil DOC and DIN are important energy and N sources for microorganisms, and

15   relative soil C and N availabilities (represented as DOC:DIN) make great contribution

to controlling microbial activity and nutrient cycling processes (Dijkstra et al., 2006;

Wu et al., 2012). Significant decrease of 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 addition increased both DOC and DIN but decreased

20   microbial biomass C (MBC) concentration; and they suggested that soil microbes

were no longer C-limited as non-significant correlation between DOC and microbial

biomass C. In their study, they failed to analyze the relationship between DOC:DIN

ratio and MBC which could be used to indicate relative C and N availabilities in determining microbial growth. In this study, coincident decrease of 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 macro- and microaggregates, respectively (Wang et al., 2016). Based on this, it was reasonable to detect significant decrease of the ratio of DOC:AP within large macro- and microaggregates (Fig. 2b). Soil acidification could enhance dissolution of phosphate from mineral-bound P pools while it was not the case for DOC (Wang et al., 2016). Thus, production of DOC might not keep up with continuous dissolution of AP resulting in lower DOC:AP ratio under N addition (Fig. 2b). Significant positive correlations between soil pH and 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, decrease of 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).

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

Within large and small macroaggregates, significant decrease of 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 (Ca > Na > Mg > K) would result in change of base cation budgets and imbalance of metal ions in soils (Lu et al., 2014). Preferential loss of exchangeable Ca relative to Mg was the main reason of significant decrease of Ca:Mg ratio in large and small macroaggregates (Fig. 3a).

5   Correlation analyses also suggested coincident decline of Ca:Mg ratio and soil pH indicating that more exchangeable Ca was leached than Mg during soil acidification in macroaggregates. However, unaffected Ca:Mg ratio in microaggregates declined our hypothesis that Ca:Mg ratio would decrease across three soil fractions. In this sandy grassland, microaggregates, containing more minerals and less sand would be a

10   more favorable site for base cation binding and adsorption which was less affected by N addition and sequential soil acidification comparing to macroaggregates (Gunina and Kuzyakov, 2014). Moreover, more decomposed SOM as indicated by δ^{13}C (Wang et al., 2015a) would provide additionally strong binding surface for exchangeable Ca and Mg in microaggregates. Thus, significant relationships of Ca:Mg ratio with soil

15   pH was not detected in microaggregates (Fig. 5c).

       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, significant decrease of Ca:Mg ratio would further influence nutrient balance, i.e. Cu uptake of plants in this calcareous

20   grassland. Researchers proposed that relative availabilities of exchangeable Ca and Mg were more important that 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 featured 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

5   role of studying soil exchangeable Ca:Mg ratio of different soil aggregate fractions in evaluating soil fertility and plant growth.

For all soil fractions, significant increase of available Fe:Mn ratio denied our initial hypothesis. Decrease of the Fe:Mn ratio might be caused the antagonistic relationship between Fe and Mn during plant uptake (Somers and Shive, 1942). Soil

10   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 productivity 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

15   acidification. Correspondingly, negative relationships of soil pH with Fe:Mn ratio (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 and 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.

20   Plant Fe deficiency was reported to induce chlorosis and decrease photosynthesis in calcareous grassland (Rogovska et al., 2007). Increase of soil available Fe:Mn ratio might pose threat to grassland productivity as potential aggravation of Fe deficiency

and occurrence of Mn toxicity to plant species (Tian et al., 2016). Soil nutrient imbalance would not only influence both quantity (yield) and quality of plants, but also cause impact on health of animals grazing on these plants (Mikha and Rice, 2004).

Conclusion

After 9-year N amendment, we found that microaggregates had lower DOC:DIN but higher DIN:AP ratio. The soil DOC:DIN ratio decreased with N addition within three fractions as a result of exogenous N supply. Nitrogen addition decreased DOC:AP

10   ratio in large macro- and microaggregates which might induce microbial C limitation relative to P. Nitrogen addition significantly decreased exchangeable Ca:Mg ratio within macroaggregates which purportly due to preferential leaching of exchangeable Ca under soil acidification as developed with N addition. Within three soil aggregate classes, significant increase of available Fe:Mn ratio might cause plant Fe deficiency

15   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.

Author contribution

Y. Jiang and Z. Xu designed the experiment; J. Yin, H. Liu, and R. Wang carried them

out. X. Feng helped to do the laboratory analyses. R. Wang prepared the manuscript with contributions from all authors.

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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 2016)).

	Bulk soil	Soil aggregates (μm)		
		> 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

Table 2 Two-way ANOVAs (*F* values) on 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^{**}

5   * Significance level at $P < 0.05$; ** Significance level at $P < 0.01$.

Fig. 1 Schematic diagram of chestnut soil illustrating three soil horizons (figure modified from Digital Science and Technology Museum of China, http://amuseum.cdstm.cn/AMuseum/agricul/6_5_27_ligt.html).
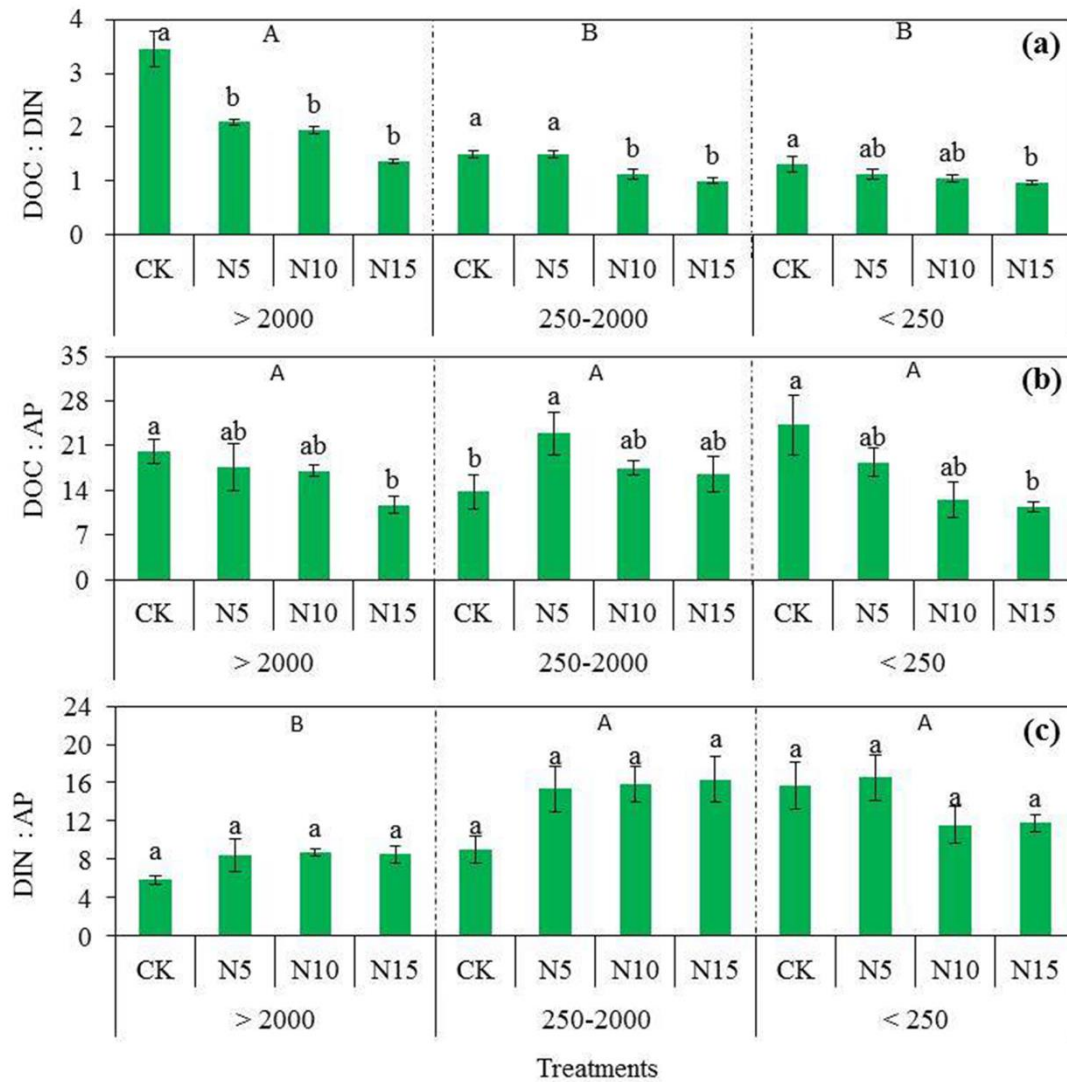
Fig. 2 The ratio of (a) dissolved organic carbon to dissolved inorganic nitrogen (DOC:DIN), (b) DOC to available phosphorus (DOC:AP), and (c) DIN:AP as influenced by N addition (0 [CK], 5 [N_5], 10 [N_{10}], and 15 [N_{15}] g N m^{-2} yr^{-1}) in soil aggregates. Data are represented as mean±SE. Lowercase 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.
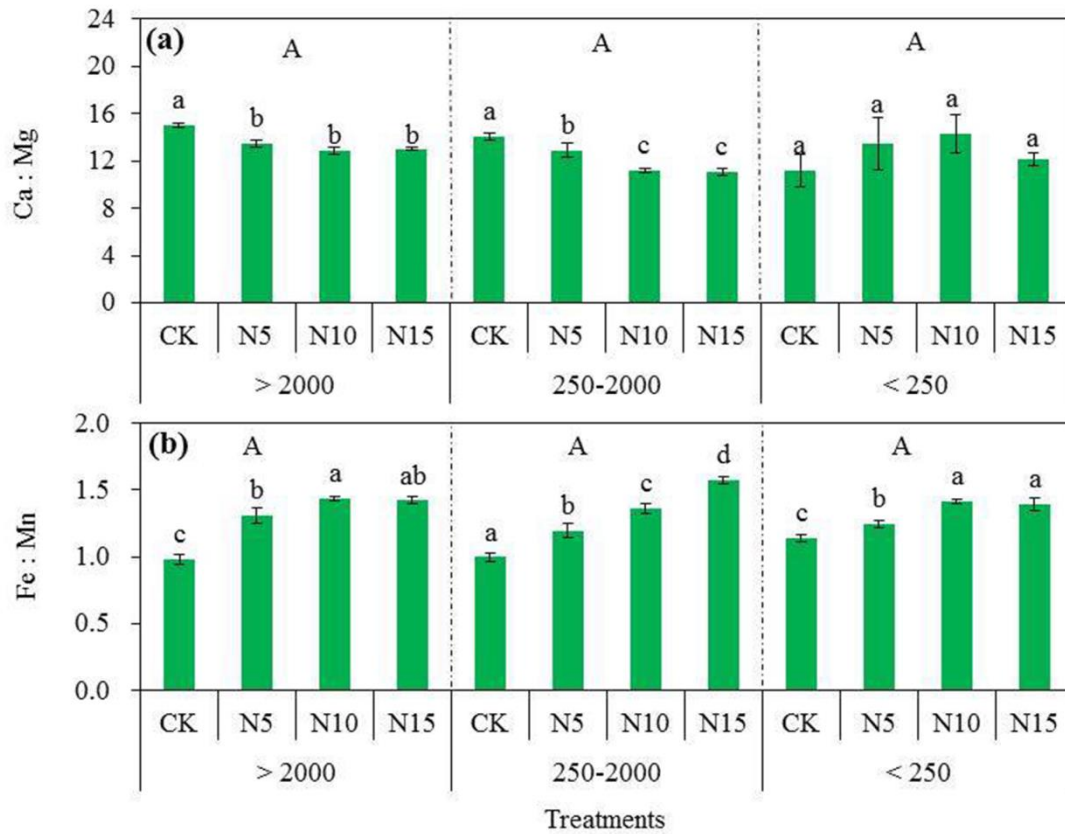
Fig. 3 The ratio of (a) exchangeable Ca:Mg and (b) available Fe:Mn as influenced by

N addition (0 [CK], 5 [N_5], 10 [N_{10}], and 15 [N_{15}] g N m^{-2} yr^{-1}) in soil aggregates.

Data are represented as mean±SE. Lowercase letters indicate significant differences

5   between N treatments within a soil fraction. The capital letters at the top indicate

significant differences between soil aggregate sizes across N treatments.
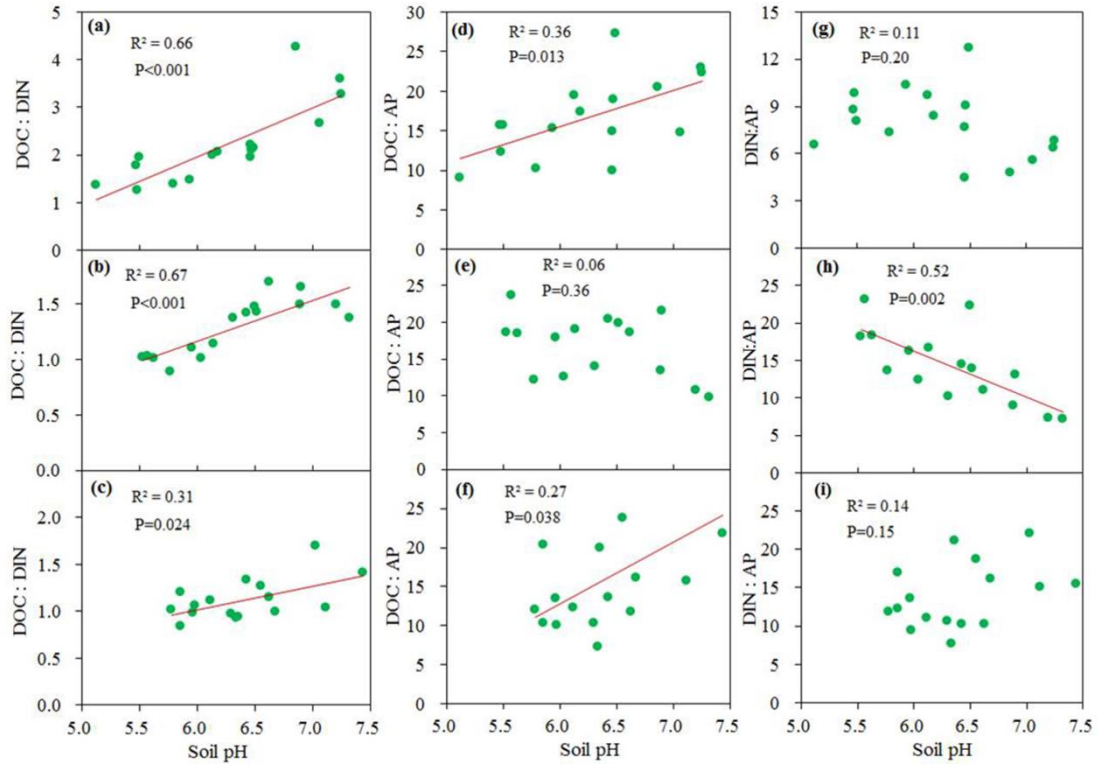
Fig. 4 The relationships between soil pH and DOC:DIN within large macroaggregates (a), small macroaggregates (b), and microaggregates (c), between soil pH and DOC:AP within large macroaggregates (d), small macroaggregates (e), and microaggregates (f), and between soil pH and DIN:AP ratio within large macroaggregates (g), small macroaggregates (h), and microaggregates (i). Significance level was set at $P < 0.05$.
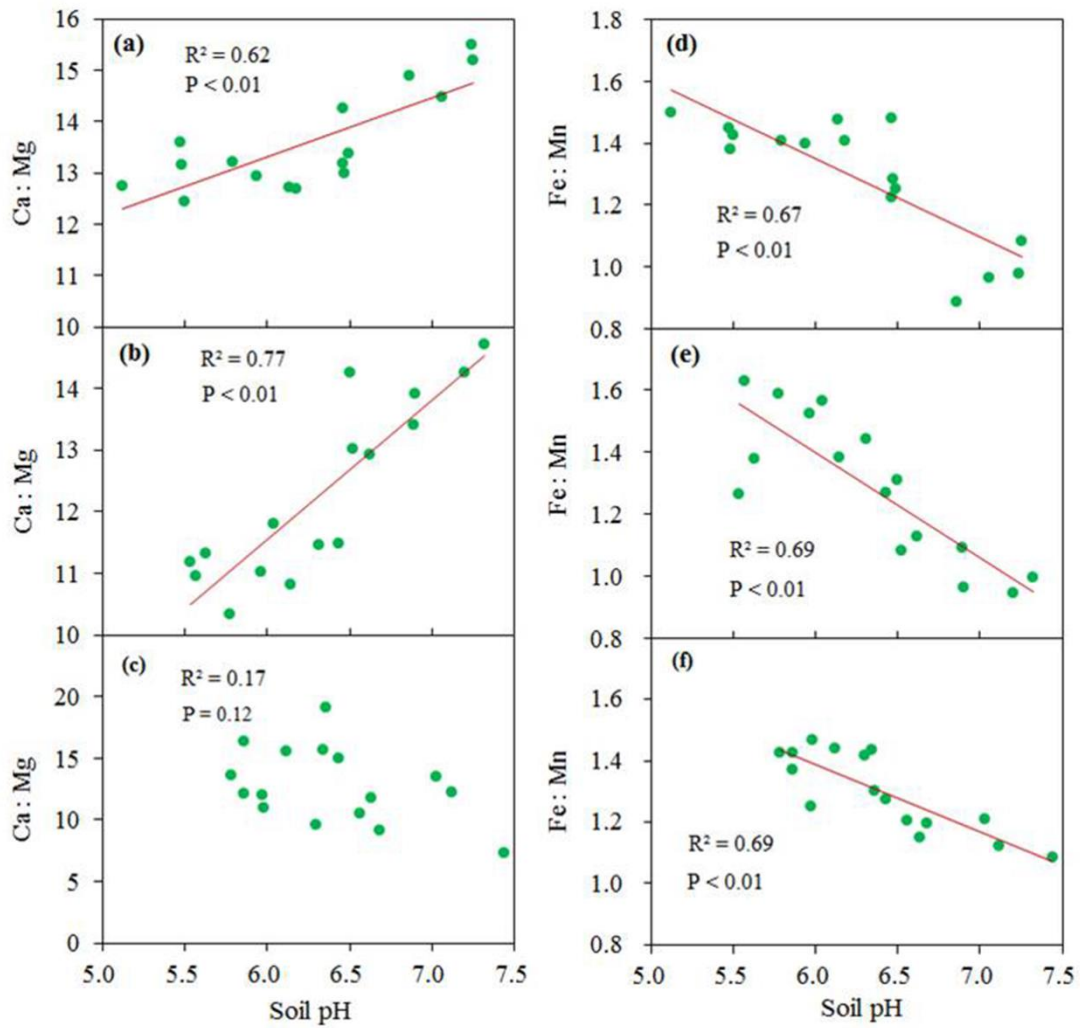
Fig. 5 The relationships between soil pH and exchangeable Ca:Mg within large

macroaggregates (a), small macroaggregates (b), and microaggregates (c), and

between soil pH and available Fe:Mn ratio within large macroaggregates (d), small

5   macroaggregates (e), and microaggregates (f). Significance level was set at $P < 0.05$.