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Effects of mineral/organic fertilisation on the soil organic nitrogen pool in a semi-arid steppe

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Abstract: Compared to solely mineral fertiliser application, organic fertiliser substitution has been demonstrated to be effective in enhancing nitrogen cycling in cropland, but the relevant research based on grassland is still insufficient. A field experiment was carried out in the grasslands of northern China to examine the impact of mineral/organic fertilisation on soil organic nitrogen fractions, activities of soil N-hydrolysing enzymes and their interrelationships. The results showed that the active soil organic nitrogen pool (hydrolysable NH_4^+-N + amino sugar-N + amino acid N) increased significantly after fertilisation, and organic fertiliser combined with mineral fertiliser addition showed the best promotion effect. N-acetyl-β-ᴅ-glucosaminidase, protease and urease activities are also significantly affected by fertilisation. The findings of our study indicate that the combination of mineral fertiliser and organic fertiliser shows a higher potential in improving the active soil organic nitrogen supply. This approach seems to be a viable agronomic strategy for augmenting soil nitrogen supply and ensuring the stability of the soil nitrogen pool in the semi-arid steppe region of northern China.

Keywords: long-term experiment; urea; organic manure; soil properties; nitrogen cycling

The Inner Mongolian grassland, which is part of the Eurasian grassland biome, holds significant economic value and ecological importance. Thus, anthropogenic activities have become increasingly significant in enhancing productivity in this area. For instance, fertilisation (Gossner et al. 2016). It is unequivocal that fertilisation would improve soil nutrient status (Tang et al. 2019). However, the excessive use of mineral fertilisers could lead to serious environmental issues, such as soil acidification and biodiversity loss (Hou et al. 2021). Compared to single mineral fertiliser application, substituting organic fertiliser is more eco-friendly. It has been proven to be a beneficial practice that can effectively increase agricultural productivity (Körschens et al. 2012, Reganold and Wachter 2016). Nevertheless, the impact of substituting organic fertiliser in grasslands remains relatively limited compared to farmlands, not to mention natural grassland that suffered less disturbance.

It is unequivocal that nitrogen (N) is a necessary limiting nutrient for plant growth and net primary productivity in grassland ecosystems. About 90% of soil nitrogen is present in organic forms. Most of the soil organic nitrogen (SON) needs to undergo decomposition to convert into available forms of nitrogen for plant absorption (Geisseler et al. 2010). The

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mineralisation of SON is facilitated by a series of various N-hydrolysing enzymes. Among these enzymes, the key ones include N-acetyl-β-D-glucosaminidase (NAG), responsible for breaking down chitin and other β-1,4-linked glucosamine polymers, urease and amidase for the liberation of ammonia from amides, and protease for the depolymerisation of proteins (Mengel 1996). Hence, enzymatic catalysis is the primary process that influences SON hydrolysis. Previous studies have indicated that different soil organic nitrogen fractions respond to fertilisation to varying degrees, such as amino acid N (Jan et al. 2009), amino sugar N (Lü et al. 2013) and hydrolysable unknown N (Xu et al. 2003). On the other hand, fertilisation was also shown to affect the N-cycling extracellular enzyme activities (Nardi et al. 2004). Previous studies pointed out that fertilisation could decrease enzyme activities involved in organic N compound degradation (protein and so on) (Ajwa et al. 1999); on the contrary, some researchers also showed that fertilisation increased N-cycling enzyme activities (Geisseler and Horwath 2008). However, previous studies mainly focused on farmland; what deserves our attention is that grassland soil ecosystems often experience unbalanced nutrient deposition input (Hou et al. 2020), so the change of N content in grassland may lead to more obvious fluctuation. Investigating the responses of soil N fractions and N-cycling enzymes to various fertilisation patterns would provide insights to facilitate the optimisation of fertilisation methods in grasslands.

As livestock grazing intensifies in China, applying mineral fertilisers becomes necessary to maintain pasture yield. However, it also leads to several issues, including nitrogen loss and environmental pollution. Organic fertiliser substitution becomes more necessary and meaningful against this backdrop. The above shows that various fertilisation patterns affect N-hydrolysing enzymes and soil N fractions separately. However, a dearth of comprehensive studies examining the response of soil N fractions and associated enzymes to various fertilisation patterns in grasslands remains. Consequently, this research aimed to investigate the aforementioned aspects: (1) the effects of mineral/organic fertiliser combinations on soil properties in grassland; (2) the effects of mineral/organic fertiliser combinations on organic N fractions and N-cycling enzyme activities in grassland, and (3) the relationships between soil N-hydrolysing enzyme activities and soil organic fractions under various fertilisations patterns in grassland.

MATERIAL AND METHODS

Experimental design and soil sampling. This study site was located in Heishantou town, Erguna city (50°12'N, 119°30'E, elevation 523 m a.s.l.), Inner Mongolia, in Northern China. The test area is located in a forest-steppe transition zone. The mean annual precipitation ranges from 200 mm to 280 mm, and the mean annual temperature ranges from –2.0 °C to 3.0 °C. The soil type in this area is classified as Chernozem soil according to the FAO classification (FAO/ISRIC/ISSS 1998). The field experiment employed a randomised block design comprising four fertilisation patterns distributed across a total of 24 blocks. The treatments are set as control (CK); urea (U); organic fertiliser (OM), and a combination of urea and organic fertiliser (U + OM). The organic fertiliser used in this experiment was organic fertiliser (cow dung). During the fertilisation process, we maintained a consistent nitrogen input, and the amount of nitrogen was 100 kg N/ha/year. Six blocks were set for each treatment as replicates (10 m × 10 m). Among the blocks, there were 1 m buffer strips. The whole experiment has been set up for eight years. Fertilisers were manually applied to the ground surface in mid-May, and soil samples were gathered in mid-August. Five random soil cores (0–10 cm depth) were extracted from each block utilising a soil auger (10 cm diameter) and subsequently combined into a single sample. After removing visible plant residues and stones, each sample underwent sieving through a 2 mm sieve. A segment of fresh soil samples was preserved at 4 °C for future laboratory assessment of soil N-cycling enzyme activities. The residual soil sample was air-dried and readied to examine soil organic N fractions and soil chemical characteristics.

Soil properties analysis. Soil pH was measured in a 1:2.5 soil/water suspension employing a digital pH meter (pH 700 Bench Meter, Eutech Instruments, Essen, Germany). Soil organic carbon (SOC) content was assessed through chemical oxidation utilising a $K_2Cr_2O_7$ solution (Nelson and Sommers 1982). Soil total N (TN) were determined by combusting ground subsamples (sieved through a 0.16 mm mesh) using an automated elemental analyser (Analyser Vario MICRO cube, Elementar, Essen, Germany).

Soil organic N fractionation. We used the acid-hydrolysis method described to analyse organic N fractions in soil (Stevenson 1996). Briefly, soil samples were heated with 20 mL of 6 mol/L HCl in an autoclave at 1 kg/cm^2 for 6 h. After Kjeldahl's digestion with

H_2SO_4 and a K_2SO_4 catalyst mixture, we determined the total hydrolysable N by steam distillation with 10 mol/L NaOH. Amino acid-N was determined by steam distillation with a phosphate-borate buffer after treatment with NaOH at 100 °C to remove amino sugar, NH_4^+, and with ninhydrin powder to convert amino N to NH_4^+. Steam distillation with MgO was used to measure hydrolysable NH_4^+-N. The quantity of N evaporated through steam distillation with phosphate-borate at pH 11.2, minus the amount of hydrolysable NH_4^+-N, resulted in amino sugar-N. The hydrolysable unknown-N was determined as the variance between total hydrolysable-N and the combined sum of hydrolysable NH_4^+, amino acid N and amino sugar N. Acid insoluble-N represented the portion of soil total nitrogen that was not accounted for in the total hydrolysable-N. Various nitrogen fraction distributions (%) were calculated as proportions of total nitrogen in the soil.

Soil N-hydrolysing enzyme activity assays. Colourimetric methods described by Landi et al. (2011) and Kandeler et al. (2011) were used to determine soil amidase, urease and protease activities. Protease activity was measured by incubating field-moist soil with sodium caseinate and 0.1 mol/L Tris (hydroxymethyl) aminomethane buffer (pH 8.1) for 2 h at 50 °C. Then, the released tyrosine was determined spectrophotometrically at 700 nm. Finally, we expressed protease activity as μg tyrosine/g soil/h (Landi et al. 2011).

We determined amidase activity by measuring the amount of ammonium released by amidase after incubating the soil with 0.1 mol/L sodium borate buffer (pH 8.5) and 0.5 mol/L formamide substrate for 2 h at 37 °C (Kandeler et al. 2011). We determined urease activity by incubating field-moist soil with a buffered urea solution (0.72 mol/L) for 2 h at 37 °C (Kandeler et al. 2011). Then, the colourimetric method at 660 nm was used to determine the released ammonium. Urease and amidase activities were expressed as μg NH_4^+/g soil/h (Kandeler et al. 2011).

Soil NAG (N-acetyl-β-ᴅ-glucosaminidase) activity was assayed by incubating 1 g soil with 4 mL of 0.1 mol/L acetate buffer (pH 5.5) and 1 mL of 10 mol/L ρ-nitrophenyl-N-acetyl-β-ᴅ-glucosaminide solutions at 37 °C for 1 h. Subsequently, 1 mL of 0.5 mol/L $CaCl_2$ and 4 mL of 0.5 mol/L NaOH were introduced to halt the reaction process. The optical density of the filtrate was quantified at 405 nm employing a spectrophotometer. Control experiments were conducted to ensure accuracy by introducing the substrate subsequent to the cessation of reactions. The soil NAG activity was determined by subtracting the control absorbance from the sample absorbance and is denoted as mg ρ-nitrophenol/kg soil/h (Parham and Deng 2000).

Statistical analysis. All values are reported based on the weight of soil after being oven-dried at 105 °C. The indexes presented in this study were the mean values of six replicates with standard errors. One-way ANOVA with a Duncan's test at the $P < 0.05$ level was conducted separately for different treatments to examine the impact of fertilisation on soil parameters. Pearson's correlation coefficients were used to investigate the correlation between soil N fractions and soil enzyme activities. All statistical analyses mentioned above were conducted utilising SPSS 16.0 software (SPSS, Chicago, USA).

RESULT AND DISCUSSION

Soil properties. Our results showed that fertilisation significantly affects soil properties, which is in accordance with previous studies. As a fundamental soil property index, U significantly decreased soil pH, whereas OM and U + OM had nearly no significant effect on soil pH compared to the control (Figure 1). This means that U accelerated the soil acidification process in grassland, while OM addition alleviated this trend. What warrants our attention is the significant increase of SOC content observed in all three treatments compared to the control, especially the U + OM treatment (Figure 1). As one of the most crucial indicators of soil fertility, increasing SOC content improves soil fertility in grasslands (Smemo et al. 2021). The soil TN content significantly increased under all three treatments compared to the control group. The U + OM treatment exhibited a more pronounced promotional effect than the other treatments (Figure 1). All fertilisation treatments had no significant effect on soil C/N compared to the control group, as illustrated in Figure 1. In summary, the U + OM treatment significantly improved soil nutrient status without causing soil acidification. It has been proven to be an excellent choice for fertilising grasslands.

Soil organic N fractions. Due to the higher availability of soil hydrolysable NH_4^+-N, amino acid-N, and amino sugar-N for soil microorganisms and plants compared to hydrolysable unknown-N and acid insoluble-N (Qiu et al. 2012), we categorised the soil organic nitrogen fractions into an active-SON pool (hydrolysable NH_4^+-N + amino acid-N + amino sugar-N)

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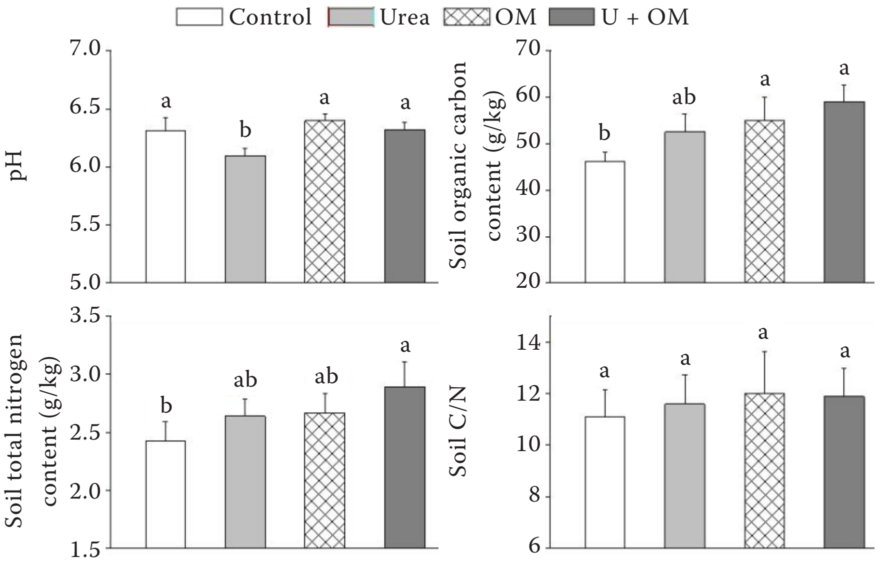

Figure 1. Soil properties under different fertilisation in soil. Different lowercase letters indicate significant differences ($P < 0.05$) among various fertilisation types in soil. Control – no fertiliser; urea – urea addition; OM – organic fertiliser; U + OM – urea combined with organic fertiliser addition

and stable-SON pool (hydrolysable unknown-N + acid insoluble-N). In terms of the soil active-SON pool, amino acid-N comprised the largest active-SON pool under various treatments about control (Figure 2), which is by a previous study (Wu et al. 2021). Our results indicated that fertilisation OM and U + OM significantly increased the amino acid-N concentration and hydrolysable NH_4^+-N (Table 1); this may be attributed to increasing N-cycling enzyme activities (protease and NAG, as observed in our experiment). The significant increase in hydrolysable NH_4^+-N due to urea addition may also be attributed to a decline in pH value. Acidification could lead to a reduction in the adsorption sites of NH_4^+ (Qiu et al. 2012). The OM and U + OM treatments led to notable enhancements in the concentration and proportion of amino sugar-N (Table 1, Figure 2). Soil amino sugar-N mainly comes from fungi cell walls. The change in this study could be attributed to the increased soil microbes after adding organic fertiliser (Wu et al. 2021). On the other hand, enhanced plant inputs, along with fertilisation, also stimulate the fungal population, leading to higher hydrolysable amino sugar-N (Tian et al. 2017).

The stable soil compound known as non-α-amino-N, which is not hydrolysable, has been identified as acid insoluble-N. This compound is considered to be composed partially of heterocyclic compounds, which are the primary structural elements of humic substances (Nannipieri and Eldor 2009). Our results showed that OM and U + OM treatments significantly increased the concentration of hydrolysable unknown-N (Table 1), which is consistent with the findings of Qiu et al. (2012) but differs from those of

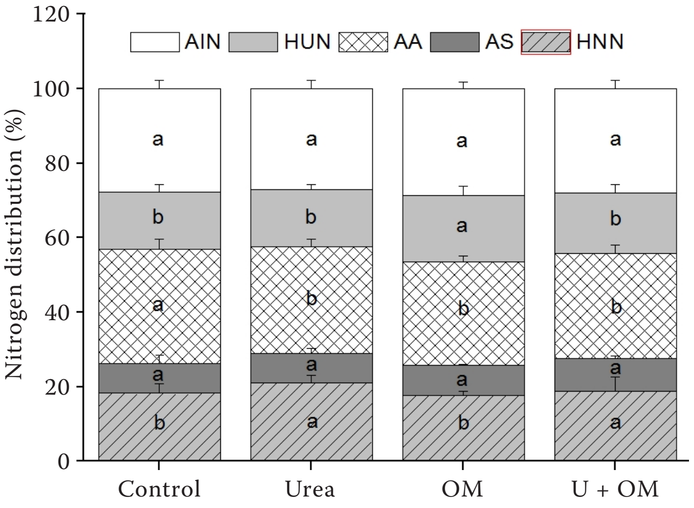

Figure 2. Distribution (%) of soil organic nitrogen under different fertilisation in soil. Different lowercase letters indicate significant differences ($P < 0.05$) among various fertilisation types in soil. AA – amino acid-N; HNN – hydrolysable NH_4^+-N; AS – amino sugar-N; HUN – hydrolysable unknown-N; AIN – acid insoluble-N; control – no fertiliser; urea – urea addition; OM – organic fertiliser; U + OM – urea combined with organic fertiliser addition

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Table 1. Concentrations (mg/kg) of soil organic nitrogen (N) fractions in soil under various fertilisation types

Type	Hydrolysable-N				Acid insoluble-N
	hydrolysable NH_4^+-N	amino acid-N	amino sugar-N	hydrolysable unknown-N	
Control	454.88 ± 44.09^b	767.06 ± 103.97^b	200.13 ± 30.4^b	386.65 ± 63.32^b	695.73 ± 69.55^b
Urea	572.88 ± 39.53^a	787.13 ± 95.97^b	216.23 ± 49.51^{ab}	416.29 ± 9.37^b	743.39 ± 74.81^{ab}
OM	513.41 ± 30.23^a	808.71 ± 32.55^a	235.11 ± 6.67^a	520.31 ± 79.24^a	835.27 ± 63.01^a
U + OM	540.12 ± 72.87^a	811.01 ± 68.56^a	251.68 ± 19.78^a	470.01 ± 85.33^{ab}	803.28 ± 73.54^a

Values in parentheses are standard errors ($n = 6$). Different lowercase and uppercase letters indicate significant differences ($P < 0.05$) among various fertilisation types. Control – no fertiliser addition; urea – single urea addition; OM – organic fertiliser addition; U + OM – urea combined with organic fertiliser application

Xu et al. (2003). Both treatments also significantly increased the proportion and concentration of acid insoluble-N significantly (Table 1, Figure 2), which may be attributed to the rise in humus content due to organic residue input (Horwath 2007). The above results indicate that the U + OM treatment could effectively stimulate the active-SON surge.

Soil N-hydrolysing enzyme activities. In our study, all applied fertilisation increased N-hydrolysing enzyme activities of NAG, urease and protease (Figure 3). This phenomenon is attributed to a higher substrate content and energy source. However, an increase was significant in all cases of NAG and urease, but only after OM aplication significantly increases of protease was noted (Figure 3). Amidase activity did not change or decrease significantly after OM application (Figure 3). It is well established that soil protease, urease, and NAG largely come from microorganisms (Kandeler et al. 2011). Abundant soil organic matter, along with fertilisation, acts as effective exogenous nutrients that enhance soil microorganisms' growth and nutrient requirements. This, in turn, stimulates the production of hydrolytic enzymes (Jia et al. 2020).

Relationships between SON fractions and N-hydrolysing enzymes. Compared to control, we mainly focused on the relationships between SON fractions and N-hydrolysing enzymes under various fertilisation treatments. Significant and positive correlations existed among soil NAG activity, amino sugar-N, and hydrolysable NH_4^+-N contents under U and U + OM (Table 2). The close association between soil N-acetylglucosaminidase (NAG) and the hydrolysis of amino sugar-N, as well as the conversion of amino sugar-N to hydrolysable NH_4^+-N through enzymatic mineralisation of SON, may explain the observed phenomenon (Wu et al. 2021). Specifically, under OM

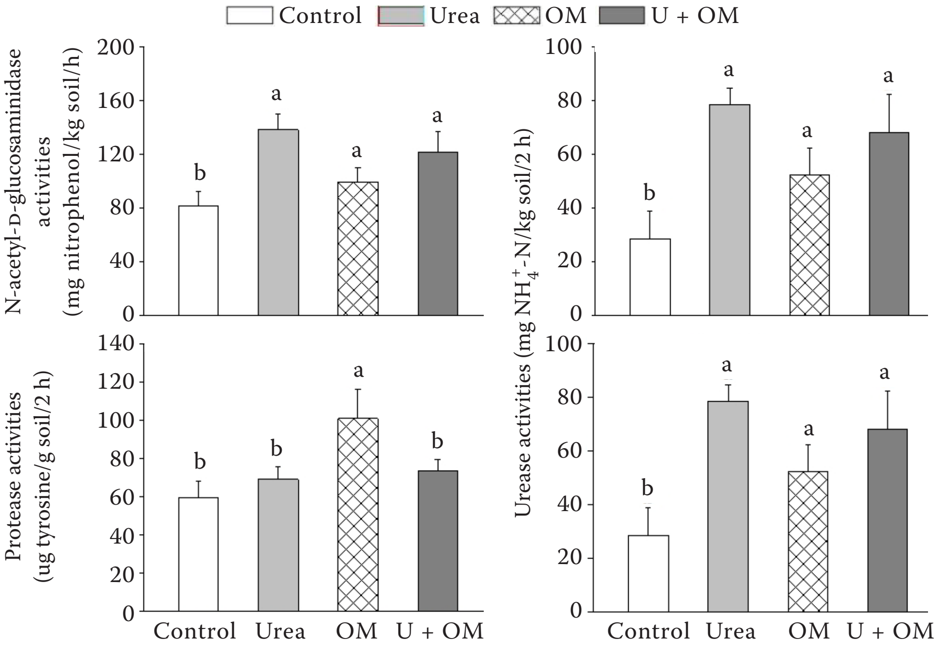

Figure 3. Soil N-hydrolysing enzyme activities are performed under different fertilisations in soil. Different lowercase letters indicate significant differences ($P < 0.05$) among various fertilisation types in soil. Control – no fertiliser; urea – urea addition; OM – organic fertiliser; U + OM – urea combined with organic fertiliser addition

Table 2. Correlations between soil organic nitrogen fractions concentrations and N-hydrolysing enzymes activities under various fertilisation types (n = 6)

Type		Amidase	NAG	Urease	Protease
Control	hydrolysable NH_4^+-N	0.851	0.543	0.312	0.647
	amino acid-N	0.88	0.377	0.897	0.234
	amino sugar-N	0.214	0.675	0.606	0.828
	hydrolysable unknown-N	0.283	0.64	0.653	0.177
	acid insoluble-N	0.511	0.566	0.371	0.92
Urea	hydrolysable NH_4^+-N	0.684	**0.907***	**0.824***	–0.631
	amino acid-N	–0.255	0.199	–0.109	0.709
	amino sugar-N	0.719	0.776	0.787	–0.712
	hydrolysable unknown-N	0.613	–0.464	–0.256	–0.347
	acid insoluble-N	–0.615	–0.097	–0.112	0.378
OM	hydrolysable NH_4^+-N	–0.547	–0.719	0.361	–0.221
	amino acid-N	–0.288	0.36	–0.035	–0.081
	amino sugar-N	0.587	–0.544	0.444	**0.982****
	hydrolysable unknown-N	0.669	0.531	–0.411	–0.034
	acid insoluble-N	–0.345	0.054	–0.443	–0.498
U + OM	hydrolysable NH_4^+-N	–0.5	**0.919****	**0.879***	–0.056
	amino acid-N	–0.791	0.43	0.573	0.539
	amino sugar-N	–0.166	**0.909***	**0.875***	–0.537
	hydrolysable unknown-N	–0.204	–0.401	–0.523	0.804
	acid insoluble-N	–0.018	0.383	0.321	0.514

**$P < 0.01$; *$P < 0.05$; NAG – N-acetyl-β-D-glucosaminidase; U + OM – urea combined with organic fertiliser addition

treatment, a significant and positive correlation was found between soil protease activity and amino sugar N content (Table 2). Most organic nitrogen associated with organic materials exists in polymer form. It should be degraded into smaller molecules to be further utilised by soil microorganisms (Nannipieri and Eldor 2009). Previous research generally pointed out that the depolymerisation of proteins into amino acids by protease is the limiting step during the soil organic N turnover process (Jan et al. 2009), so it is reasonable that protease plays an important role. However, no significant relationships were observed between stable-SON (acid insoluble-N + hydrolysable unknown-N) and N-mineralising enzyme activities under various fertilisation treatments (Table 2).

Based on a long-term fertilisation experiment in a semi-arid grassland, our results indicate that fertilisation significantly accelerated the enzymatic turnover process of soil organic N fractions in grassland. Additionally, differences were observed in this process among various fertilisation patterns. Our findings also suggest a strong correlation between alterations in SON fractions and the activities of nitrogen-hydrolysing enzymes. Combining mineral fertiliser with organic fertiliser led to heightened levels of soil N-hydrolysing enzymes and concentrations of active SON fractions. Consequently, integrating mineral and organic fertilisers appears to be a viable agronomic approach for improving soil N availability and preserving the stability of the soil N pool in the semi-arid area of Northern China.

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