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Impacts of Elevated CO_2 on Nitrogen Cycling and Isotopic Composition in the Alfalfa-Soil System

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Abstract

Purpose: The increase in CO_2 concentration significantly impacts the nitrogen cycle dynamics which is closely related to plant growth dynamics and can change microbial activities in terrestrial ecosystems. However, this is dependent on environmental factors affecting soil types, plant types and microbial systems etc. In this study, the effect of elevated CO_2 concentration on changes in nitrogen isotopic composition in soil and leaves of alfalfa, a nitrogen-fixing plant, and the response of the nitrogen cycle process are studied in the Loess Plateau of China.

Methods: Hexagonal open-top chambers were set up in the experimental area. The automatic control system was adopted to adjust the rate of CO_2 spraying. Alfalfa seeds (seedling) (20 seeds (seedlings) per pot) were planted and 50g of leaves were collected. Soil samples were pretreated before planting. The samples were washed and deactivated for determination of total nitrogen content and $\delta^{15}N$ value. The content of NO_3-N and NH_4^+-N in soil were determined. The NO_3^--N and NH_4^+-N contents were also determined. The ^{15}N isotope values in soil and plant leaves were pre-treated and then $\delta^{15}N$-NO_3^- was determined. The experimental data were initially organized and analyzed using Microsoft Excel 2019 and statistical analysis was performed using SPSS 27.0.

Results: The results showed that under the semi-arid climate conditions of the Loess Plateau, with the increase of CO_2, total inorganic nitrogen in soil decreased, the nitrate-nitrogen concentration and the $\delta^{15}N$ are negatively correlated with the CO_2 concentration. It shows that with the increase of CO_2, both nitrification and denitrification in the soil will weaken with the decrease of microorganisms. However, the degree of denitrification weakening is greater than nitrification. The nitrogen level in alfalfa leaves gradually increased with the increase in CO_2 concentration.

Conclusion: This indicated that the rise in CO_2 concentration could promote alfalfa's nitrogen absorption and improve alfalfa's nitrogen utilization efficiency. This study provides a scientific basis for changes in the nitrogen cycle process of soil and plant in the Loess Plateau under the greenhouse effect.

Keywords: nitrification, denitrification, δ15N, nitrogen cycle, soil and plant systems, the Loess Plateau.

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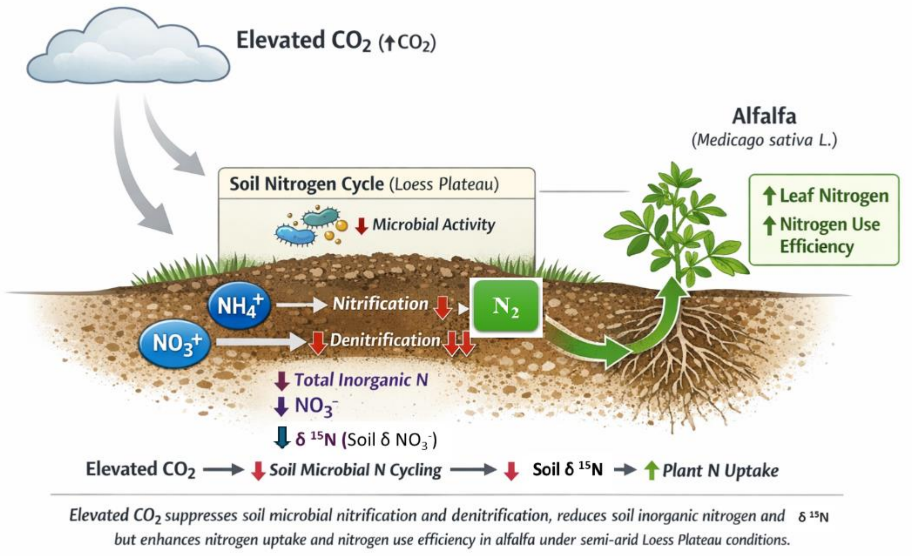
Graphical abstract



1. Introduction

The current global warming situation is severe, and the increase of CO_2 concentration is an important factor of global environmental change (Shah *et al.* 2024). According to data, as of 2024, the global average CO_2 concentration has reached 427.09ppm (https://www.co2.earth/). According to (Bai *et al.* 2024), atmospheric CO_2 concentrations are now higher than they have been in the last 26 million years and are predicted to nearly double by the end of this century. After increasing since the pre-industrial era, atmospheric CO_2 concentrations are predicted to reach 720-1000 ppm, which will help raise global air temperatures from 2.6 to 5.4°C by the end of the 21st century (Sharma and Singh 2021). The structure, function, and overall productivity of the vegetation system are expected to be impacted by the increase in atmospheric CO_2 concentration causing climate change (Shah *et al.* 2022, 2023). In general, when adequate resources like soil nitrogen (N), water, and favorable temperatures are available, elevated atmospheric CO_2 stimulates photosynthesis mechanisms, which improves biomass production and yields (Parkash *et al.* 2022). Recent literature demonstrates that climate change has extensive environmental and socio-economic effects that are not limited to the ecosystem processes. Extremes in climate may exacerbate energy insecurity and economic cost by disrupting infrastructure and altering prices (Lei and Xu, 2025; Wu *et al.*, 2025). Meanwhile, climate governance policies and climate risks have been demonstrated to improve corporate environmental responsibility and green innovation via institutional, financial, and reputational channels (Lei and Xu, 2025; Wang *et al.*, 2025). Moreover, the digital economy also helps to improve the environment by introducing green innovation and structural change, which facilitates more comprehensive transitions to sustainability (Tian *et al.*, 2025). Combined, these findings underscore the fact that climate risks exist at environmental, economic, and governance systems. Nevertheless, only a few studies have attributed such macro-level climate effects with ecosystem-level nitrogen cycling feedbacks to higher atmospheric CO 2, which is still vital in interpreting biogeochemical feedbacks and sustainability consequences. Since CO_2 is involved in the photosynthesis of plants, the increase of CO_2 concentration will have an impact on the process of photosynthesis of plants, thus affecting the growth of plants and their absorption of nitrogen (Kizildeniz 2024). The increased CO_2 concentration had a significant effect on soil nitrogen cycle (Davidson *et al.* 2008; WIEDER *et al.* 2011), nitrification, and denitrification play a key role in the entire soil nitrogen cycle (Liu *et al.* 2011). Therefore, improving our understanding of the relationship between increased CO_2 concentration and the nitrogen cycle of the ecosystem is conducive to a better prediction of the future ecosystem nitrogen cycle.

The effect of elevated CO_2 concentration on nitrogen changes in soil and plants has attracted extensive attention. N is one of the vital elements for plant growth (Kuypers *et al.* 2018; Ren *et al.* 2014; Zhang *et al.* 2015) compared with organic N, NO_3^--N and NH_4^+-N are the two main sources of mineral nitrogen that can be absorbed and utilized during plant growth. Dong *et al.* (2020); and Luo *et al.* (2019) studied the effect of elevated CO_2 concentration in greenhouse soil on nitrogen uptake and the cycle of cucumber plants. They found that an appropriate increase in CO_2 concentration could improve the nitrogen uptake efficiency of cucumber fine roots and reduce soil nitrogen loss. Bloom *et al.* (2010) investigated the effect of elevated CO_2 concentration on the nitrogen status of wheat and Arabidopsis. They found that increased atmospheric CO_2 reduced the photorespiration rate in plants, which subsequently prevented nitrate from being assimilated into organic nitrogen compounds. This reduction in nitrate assimilation likely occurs because photorespiration provides key intermediates and energy required for nitrate assimilation. Thus, under higher CO_2 concentrations, the reduced photorespiration disrupts this process, affecting the conversion of nitrate into plant material.

The studies discussed here show the varied effects of elevated CO_2 concentrations in the perspective of plant physiology. However, investigating the effect of CO_2 on the nitrogen cycle through measurements of the $\delta^{15}N$ value offers advantages over traditional methods, which may not effectively capture such dynamics (Liu *et al.* 2017). ^{15}N natural abundance as a comprehensive index of the ecosystem nitrogen cycle can not only clarify the process of nitrogen migration and transformation but also effectively indicate the level of nitrogen limitation and nitrogen release in the ecosystem (Hobbie and Högberg 2012; Pardo *et al.* 2006). The plant $\delta^{15}N$ value indicates the available nitrogen level of the ecosystem, the N source absorbed by the plant, the N conversion rate of the ecosystem, and the dependence of the plant on mycorrhizal fungi. The soil $\delta^{15}N$ value can reflect the N cycle on a larger time scale. The change of N isotopes can better indicate the process of the N cycle and the different degrees of isotopes fractionation. Thus, we can better understand nitrogen fixation in terrestrial ecosystems. The nitrogen isotope values of plants and soil are used as indicators of the terrestrial nitrogen cycles, which can reflect the response of the terrestrial ecosystem nitrogen cycle to global change (Houlton *et al.* 2006; Robinson 2001).

The nitrogen isotope value of the soil is determined by the N input process, and the output process, and the corresponding fractionation during the nitrogen migration process (Fang *et al.* 2013). The nitrogen isotope values of soil and plants are affected by various external factors, such as temperature, precipitation, atmospheric nitrogen deposition, and climatic conditions. Whether in local or global scope, the most important is the influence of climatic conditions (Amundson *et al.* 2003; Zhang *et al.* 2016, 2015). However, in the context of global CO_2 increase, the mechanism of the nitrogen cycle and nitrogen isotope change in soil and plants has not been recognized uniformly. Some studies suggested that increasing atmospheric CO_2 concentration would reduce the nitrogen leaching and denitrification, increasing the nitrogen fixation in the ecosystem (Cannell and Thornley 1998; Hungate *et al.* 1999). Other studies also showed that elevated CO_2 concentration would increase the denitrification of soil, and at the same time increase the release of N_2O, thus reducing the retention of nitrogen in the ecosystem (Barnard *et al.* 2005). Therefore, the experimental conclusions are quite different under different experimental scenarios, depending on the specific soil-plant system. In particular, it is not clear that when the CO_2 concentration increases, in the soil-plant system under semi-arid climatic conditions in the Loess Plateau, the nitrogen content and stable nitrogen isotope changes in the soil and plant leaves and the response of the nitrogen cycle process are not clear.

Alfalfa is a forage planted widely in the world. It is a naturally distributed high-yield, cold-tolerant, and drought-tolerant legume plant. It is also a C_3 plant (the initial product of CO_2 assimilation is a three-carbon compound in the photosynthetic carbon cycle-Phosphoglyceric acid plants), can go deep underground, have nodules, can provide nitrogen nutrition for roots, increase soil nitrogen input, and have a significant response to changes in CO_2, so this study chose alfalfa as a test plant (Kizildeniz 2024; Yang *et al.* 2019). In this study, we measured both $\delta^{15}N$ and $\delta^{18}O$ values in the samples. While $\delta^{15}N$ provides insights into nitrogen cycling processes and isotopic fractionation during transformations such as nitrification and denitrification, $\delta^{18}O$ in nitrate (NO_3^-) serves as an additional tracer. Measuring $\delta^{18}O$ helps distinguish between sources of nitrate (e.g., atmospheric deposition vs. microbial processes) and provides complementary information on nitrate dynamics in the alfalfa-soil system under elevated CO_2 conditions. This dual-isotope approach enhances the resolution of nitrogen cycling pathways and their response to environmental changes.

The study focuses on the changes of nitrogen content and stable nitrogen isotope in soil and plant leaves, as well as the response of nitrogen cycling process in soil and alfalfa soil-plant system under semi-arid climatic conditions on the Loess Plateau under three different CO_2 concentrations namely ambient, 500ppm and 700ppm. And this paper measured NO_3^--N, NH_4^+-N, total dissolved nitrogen (TDN), and $\delta^{15}N$ isotopic values by setting three CO_2 concentrations, namely ambient CO_2, 500 ppm CO_2, and 700 ppm CO_2. According to the variation trend of various results, the possible causes were analyzed from the perspective of isotope fractionation, aiming to determine the effect of the change of CO_2 concentration on the nitrogen cycle in soil and plants.

2. Material and methods

2.1. *Study site*

The experimental site is located at the Weishui Campus (34°37′N, 108°91′E) of Chang'an University, Xi'an, Shaanxi Province, China. It has a temperate monsoon climate with an average annual temperature of 13.7 degrees Celsius, characterized by hot, humid summers and cold, dry winters. Precipitation levels are moderate, but the area experiences water scarcity and frequent periods of drought, making it generally semi-arid.

2.2. *Experimental design*

In the experimental area hexagonal open-top chambers were set up (diameter:4.4 m, high:1.6m). Each chamber has similar environmental conditions (light, microorganism, temperature, etc.). There are six identical open-top chambers in total, every two chambers for the same CO_2 concentration in parallel, and each air chamber includes two same processing, a total of four times repeated. Since the global average CO_2 concentration in recent years has reached more than 400 ppm. Three levels of CO_2 treatments were set, namely 400 ppm (ambient), 500 ppm, and 700 ppm. To maintain the target levels of CO_2 in the open-top chambers, an automatic control system was linked to an infrared gas analyzer (IRGA) to control the CO_2 levels in the chambers. The concentrations of CO_2 were measured and recorded at regular intervals and the system automatically adjusted the injection of CO_2 in case of variation in the set concentrations. The measured CO_2 concentrations were near the target values with slight variations around the set points during the 3.5-month growth period. To guarantee the spatial homogeneity, the CO_2 concentrations were measured periodically in several positions in each chamber and it was proved that the CO_2 was distributed rather homogeneously. Several pots of plastic tanks (length 70cm, width 40cm, height 50cm) filled with soil in each air chamber (**Figure 1**), the soil type used in the experiment is luvisols. After mixing it, adding the same quality soil (about 20kg) into each plastic tank to ensure the same soil properties in each pot. Alfalfa seeds were planted on 23 July 2019, about 20 seedlings per pot were planted. With a growth period of 3.5 months, after the growth period was over, about 50g of leaves were collected from each plastic pot for subsequent treatment.

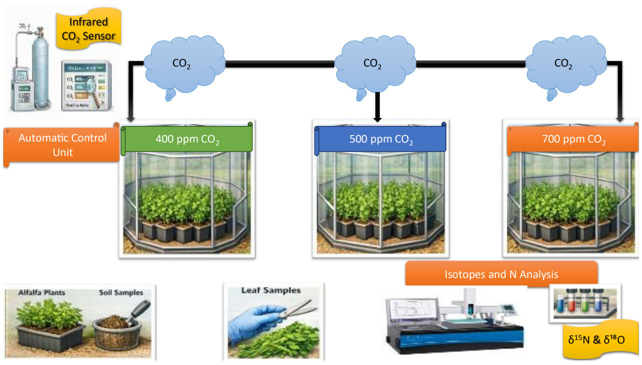


Figure 1. Experimental design, open-top chambers setup.

2.3. *Sample pretreatment*

Soil samples were collected before planting alfalfa, the soil at 0~10 cm depth was sampled, and the coarse roots and stones were removed. Then immediately passed through a 5mm sieve, divided into two samples, one was dried in an oven to constant weight at 105°C for moisture content and extractable nitrogen analysis, and the other sample was used to determine inorganic nitrogen. After collecting alfalfa samples, some samples were stored at -18°C before treatment to determine nitrate and ammonium nitrogen. For plant leaves remaining samples were washed and deactivated at 105°C for 30min and dried to constant weight at 65°C for determination of total nitrogen content and $\delta^{15}N$ value.

2.4. *Sample analysis*

The soil was extracted with 2M KCL (soil-liquid ratio: 1:5), shaken for 1h, and left to stand for 30min. After standing, the soil was filtered through a 0.45 µm syringe filter. The moisture content of the soil is obtained by dividing the difference between fresh weight and dry weight by dry weight. The content of NO_3-N in soil was determined by ultraviolet spectrophotometry, the content of NH_4^+-N in soil was determined by the indophenol blue colorimetric method, and the TDN in soil was analyzed by TOC analyzer. After digesting the plant leaves with sulfuric acid-hydrogen peroxide (Wu *et al.* 2000), ultraviolet spectrophotometry, and indigo blue colorimetry were also used to determine the NO_3^--N and NH_4^+-N content in the plant leaves. The ^{15}N isotope values in soil and plant leaves are pre-treated with the bacterial denitrification method, and then the $\delta^{15}N$-NO_3^- is determined using ^{15}N-gas chromatography mass spectrometry (GC-MS). The results are corrected using international standard samples, and the stable isotope ratio is expressed as δ as follows:

$$\delta(0/00) = (R_{sample}/R_{standard} - 1) * 1000 \quad (1)$$

In the formula, the R sample and the R standard are the sample and the standard $^{15}N/^{14}N$ or $^{18}O/^{16}O$, respectively.

2.5. *Statistical Analysis*

The experimental data were initially organized and analyzed using Microsoft Excel 2019. Statistical analysis including, one-way ANOVA followed by Tukey's HSD test, was performed and experimental data graphs were generated using SPSS 27.0.

3. Results

3.1. *Change of nitrate, ammonium, and dissolved total nitrogen content in the soil*

Compared with the ambient CO_2, when the concentration of CO_2 increases, the ammonium nitrogen content in the soil shows different degrees of increase, with an average increase of 71% and 4% under 500 ppm and 700 ppm CO_2 (**Figure 2**). Under two kinds of elevated CO_2 concentration can be observed at the content of NO_3^--N and TDN show significant decreases, and among them, the concentration of 500ppm CO_2 reduces the NO_3^--N and TDN by 44% and 20% respectively, while at 700ppm CO_2, respectively with a reduction of 68% and 42% (**Figure 2**), the NO_3^--N and TDN content at 700 ppm CO_2 is much lower than those of 500 ppm CO_2, and the impact is more significant indicating a stronger inhibitory effect at higher CO_2. Overall, when the CO_2 concentration increases, the nitrogen level in the soil shows a downward trend, and the decline is observable. Statistical analysis indicated that CO_2 concentration had a significant effect on all variables ($P < 0.001$). Tukey's test showed that NH_4^+-N at 500 ppm was higher than at 400 ppm ($P = 0.002$) and at 700 ($P= 0.016$), while 400 ppm and 700 ppm did not differ ($P= 0.408$). For NO_3^- -N and TDN, all pairwise differences were significant ($P< 0.001$)

3.2. *Changes of nitrogen and oxygen isotope values in soil*

According to the bar chart in **Figure 3**, the nitrogen and oxygen isotopes in the soil both show a downward trend as the CO_2 concentration increases. Compared with ambient (400 ppm) CO_2, at CO_2 concentrations of 500 ppm and 700 ppm reduced the nitrogen isotope values in the soil from 11.6‰ to 9.9‰ and 6.6‰, and oxygen isotope values from 11.3‰ to 8.8‰ and 6.7‰, respectively. The changing trend of nitrogen and oxygen isotopes in soil was the same as that of $\delta^{15}N$-NO_3^- described in **Figure 2**, and the declining trend brought by 700ppm CO_2 was more significant. One-way ANOVA confirmed that CO_2 concentration had significant effect on both nitrogen and oxygen isotopes ($P < 0.001$). Tukey's HSD test indicated that all pairwise differences among treatment were significant $P < 0.001$).

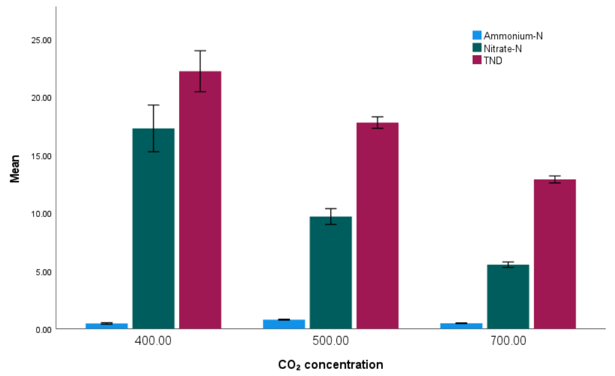


Figure 2. Effects of CO_2 concentration on NH_4^+-N, NO_3^--N, and TDN in soil. Values are mean ± SE (n = 4). One-way ANOVA and Tukey's HSD, ($p < 0.05$) indicate significant differences among treatments.

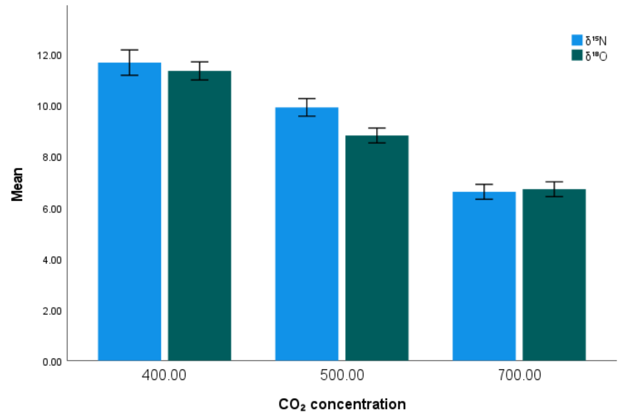


Figure 3. Effect of CO_2 concentration on nitrogen and oxygen isope values in soil. Values are mean ± SE (n = 4). One-way ANOVA and Tukey's HSD, ($p < 0.05$), indicate significant differences among treatments.

3.3. *Changes of ammonium nitrogen and nitrate nitrogen content in plant leaves*

Compared with the changing trend of nitrogen level in soil, the changing trend of inorganic nitrogen and isotope in alfalfa leaves showed different trends. With the constant increase of CO_2 concentration, ammonium nitrogen content generally increases first and then decreases. However, compared with atmospheric CO_2 concentration, the content of ammonium nitrogen under 500 ppm and 700 ppm shows different proportions of increase, 18% and 10% respectively. However, the changing trend of NO_3^--N content in leaves differed from that of ammonium nitrogen. With the increase of CO_2 concentration, NO_3^--N content also showed a constant increase. The NO_3^--N under 500 ppm and 700 ppm CO_2 is higher than that under ambient CO_2, increased by 12% and 15% respectively (**Figure 4**). Statistical analysis indicated that CO_2 concentration had significant effect on both ammonium nitrogen and nitrate nitrogen ($P < 0.001$). Tukey test indicated that the ammonium nitrogen showed significantly differences among all treatments, (400 vs 500 ppm), $p < 0.001$; 400 vs 700 ppm, $p < 0.001$; 500 vs 700ppm, $p = 0.004$). For nitrate nitrogen, the values at 500 ppm and 700 ppm were both higher significantly than those at ambient conditions ($p < 0.001$), while no significant difference was observed between 500 ppm and 700 ppm ($p = 0.132$).

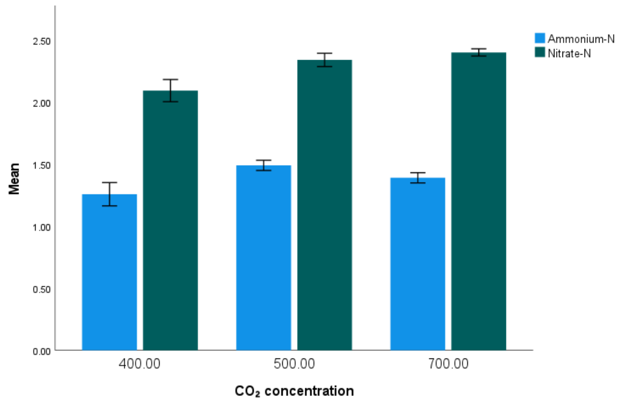


Figure 4. Effects of CO_2 concentration on NH_4^+-N and NO_3^--N, in plant leaves. Values are mean ± SE (n = 4). One-way ANOVA and Tukey's HSD, ($p < 0.05$), indicate significant differences among treatments.

3.4. *Changes of nitrogen and oxygen isotope values in plant leaves*

The value of ^{15}N increased at both elevated CO_2 concentrations, the value under 500 ppm CO_2 increased from 0.6‰ at the ambient condition to 2.5‰, while the value under 700ppm CO_2 increased from 0.6‰ to 1.2‰, but decreased from 2.5‰ under 500ppm CO_2 to 1.2‰. But overall, it has the same increasing trend as ammonium nitrogen, and we can find that the $\delta^{15}N$ in plant leaves is much lower than that in soil. On the contrary, the $\delta^{18}O$ value showed a downward trend with increased CO_2 concentration. The value 17.7‰ at ambient condition decreased to 10.4‰ at 500 ppm, and 12.2‰ at 700 ppm. Although a slight increase observed at 700 ppm compared with 500 ppm, but remained clearly lower than at ambient CO_2 (**Figure 5**). Statistical analysis showed that CO_2 concentration had significant effect on both $\delta^{15}N$ and $\delta^{18}O$ ($p < 0.001$). Tukey test indicated that all pairwise differences among treatments were significant ($p < 0.001$)

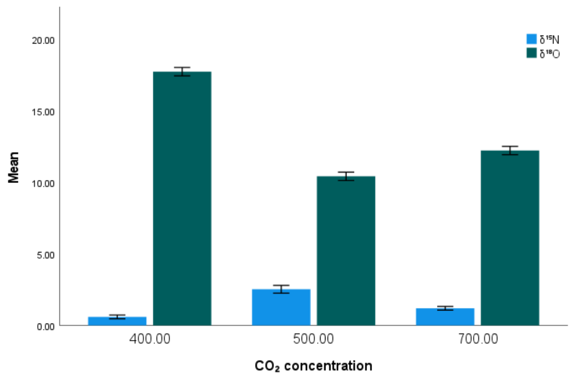


Figure 5. One-way ANOVA revealed that CO_2 concentration had a significant effect on $\delta^{15}N$ and $\delta^{18}O$ values in plant leaves ($p < 0.001$). Tukey's HSD test showed that all treatments differed significantly from each other ($p < 0.05$).

3.5. *Variation in Total Inorganic Nitrogen (TIN) Contents in Soil Under Different CO_2 Concentrations*

Figure 6 presents the variation in total inorganic nitrogen (TIN) contents in soil under different CO_2 concentrations; ambient, 500 ppm, and 700 ppm. The TIN values are measured in $mgkg^{-1}$ of soil, with error bars representing the standard errors of the means. With increasing CO_2 concentration, TIN content in soil showed a clear consistent decreasing trend. The data suggests how elevated CO_2 concentrations influence nitrogen dynamics in the soil, potentially altering microbial activity, nitrogen fixation, and plant nitrogen uptake. The error bars help assess the precision of the measurements, with larger bars indicating more variability. The differences in TIN contents between the treatments provide insights into the impact of higher CO_2 concentrations on soil nitrogen cycling processes such as nitrification and denitrification. Statistical analysis indicated that CO_2 concentration significantly effected TIN content ($p < 0.001$). Tukey's HSD test confirmed that all pairwise comparisons among treatments were significant ($p < 0.001$).

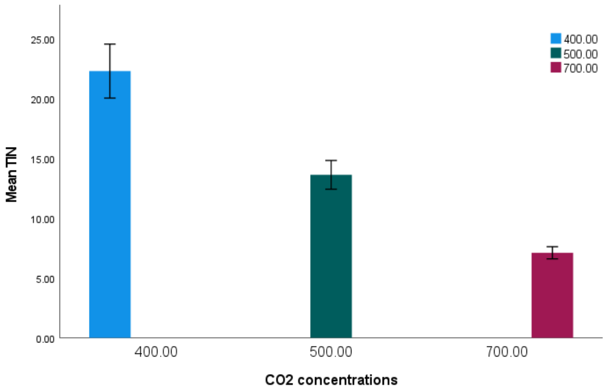


Figure 6. Means of TIN contents in soils under different CO_2 concentration. Values are mean ± SE (n = 4). One-way ANOVA and Tukey's HSD, ($P < 0.05$). indicate significant differences among treatments.

4. Discussion

4.1. *Nitrogen Cycle and Isotope Fractionation*

The table summarizes fractionation factors for key nitrogen cycle processes, reflecting how each step discriminates between nitrogen isotopes (^{14}N and ^{15}N) and influences isotopic compositions ($\delta^{15}N$) in ecosystems. Biological nitrogen fixation shows minimal fractionation, as microbes use atmospheric N_2 with little preference for lighter isotopes. Ammoniation, the breakdown of organic nitrogen to ammonium, involves no fractionation, while ammonium volatilization and nitrification exhibit significant fractionation due to microbial or chemical preferences for ^{14}N. Denitrification, where nitrate is reduced to gaseous nitrogen forms, shows variable fractionation depending on substrate availability. Plant assimilation also discriminates against heavier isotopes, with the degree of fractionation influenced by nitrogen sources and environmental conditions. These factors are vital for tracing nitrogen transformations and understanding ecosystem dynamics under various environmental influences.

It can be Observed that the $\delta^{15}N$ values in alfalfa leaves were markedly lower than those in soil across all CO_2 treatments. This consistent isotopic offset likely reflects the contribution of biological nitrogen fixation (BNF) via root nodules. As a legume, *Medicago sativa* forms symbiotic associations with rhizobia that fix atmospheric N_2, which has a $\delta^{15}N$ value near 0‰ due to minimal isotopic fractionation during fixation. Consequently, greater reliance on fixed nitrogen results in lower foliar $\delta^{15}N$, whereas greater uptake of soil-derived nitrogen leads to higher $\delta^{15}N$ values. This mechanism provides a plausible explanation for the observed soil-plant isotopic differences in the present study. Furthermore, elevated CO_2 can enhance photosynthesis and increase carbon allocation to nodules, potentially stimulating nitrogen fixation activity and promoting the assimilation of isotopically lighter nitrogen. Such CO_2 driven shifts may therefore help explain the patterns of leaf $\delta^{15}N$ observed under elevated CO_2 conditions.

Table 1. Nitrogen cycle and nitrogen isotope fractionation coefficient in ecosystem (Hogberg 1997).

Nitrogen process	Fractionation factor
Biological nitrogen fixation	0.998-1.020
Ammoniation	1.000
Ammonium volatile	1.029
Nitrification	1.015-1.035
Denitrification	1.000-1.033
Plant assimilation	0.980-1.020

4.2. *Effects of Elevated CO_2 on Soil Nitrogen Dynamics and Isotopic Tracers in the Loess Plateau: Insights into Nitrification and Denitrification*

As shown in **Figure 6**, when the CO_2 concentration increased, the content of TIN in the soil decreased significantly. This is mainly due to the effect of CO_2 concentration change on soil microbial activity. With the increase of CO_2 concentration, the soil C content has changed accordingly. CO_2 is the main influencing factor of soil microbial activity and changes the structure of the soil microbial community. Studies have shown that when the CO_2 concentration increases, the gene abundance of some soil bacteria tends to decrease, such as the ammonia monooxygenase encoding gene *amoA*, which are involved in the process of soil ammonia oxidation and can catalyze the ammonia oxidation process, and the denitrifying bacteria *nirS* and *nirK* (He *et al.* 2013). This weakens the soil nitrification and denitrification process, which leads to a decrease in the TIN content generated by soil organic nitrogen degradation.

Second, the soil's ammonium nitrogen content progressively rose as the CO_2 concentration rose, despite the fact that the TIN content showed a declining tendency. Tscherko *et al.* (2001) believe that this is due to the increase of available carbon content for decomposition and utilization by microorganisms, which leads to the enhancement of ammunition in the soil. In addition, the increase in CO_2 concentration is possible reason for the increase in ammonium nitrogen content in the soil, which resulted in a significant decrease of NO_3^--N content and an increase of ammonium nitrogen content in the soil.

With the increase of CO_2 concentration, the NO_3^--N in soil decreased significantly. Studies have shown that the increase in CO_2 concentration increases the soluble C entering the soil, which in turn promotes the hetero-oxygen fixation of soil available nitrogen by soil microorganisms, thereby reducing the substrates in the soil nitrification process, and ultimately resulting in a decrease in the concentration of soil NO_3^--N (Cookson *et al.* 2005; Drake 2013; Hungate *et al.* 1999). However, the decrease of nitrate activity in the soil caused by increased CO_2 concentration will also lead to the decrease of NO_3^--N concentration in soil (Barnard *et al.* 2005). In addition, through meta-analysis, Dai *et al.* (2020) found that regardless of whether there are plants in the soil, the increase in temperature will increase the concentration of NH_4^+ and dissolve organic nitrogen (DON) in the soil, and promote the transformation of microbial nitrogen cycle from anabolic process to catabolic process. Less nitrogen is converted into microbial biomass nitrogen, while a relatively large proportion of organic nitrogen is released in the form of NH_4^+ through nitrogen mineralization, increasing the availability of inorganic nitrogen (NH_4^+) in the soil environment (Dai *et al.* 2020). The increase of CO_2 concentration is closely related to the rise of temperature (IPCC Working Group 1 *et al.* 2013). However, the real-time soil temperature under different CO_2 concentrations was not monitored in this paper. Therefore, the influence of elevated CO_2 and temperature on soil microbial nitrogen cycle from anabolism to catabolism cannot be quantitatively studied. This requires further studies to further relate the quantitative relationship between CO_2 concentration and temperature as well as the influence on microbial nitrogen cycling.

Soil nitrogen cycle processes in terrestrial ecosystems are affected by microorganisms. Different microorganisms regulate different nitrogen cycle processes, resulting in different nitrogen isotope fractionation characteristics. The nitrogen conversion processes of percolation, nitrification, and denitrification are accompanied by strong nitrogen isotope fractionation, resulting in ^{15}N enrichment left in the soil nitrogen pool and ^{15}N depletion lost from the system (Vallano and Sparks 2012). The fractionation effect in the process of biological nitrogen fixation and soil organic nitrogen mineralization is relatively small, and its $\delta^{15}N$ is close to that of the atmosphere. **Table 1** lists the fractionation coefficients of each process in the nitrogen cycle of the ecosystem. The higher the fractionation coefficient, the more obvious the fractionation effect, and the more significant the ^{15}N enrichment of the substrate (HÖGBERG 1997).

The experimental results of this study showed that both NO_3^--N concentration and $\delta^{15}N$ value in the soil were negatively correlated with CO_2 concentration. Soil nitrate (NO_3^-) is produced through nitrification and consumed during denitrification, two processes that exert opposing effects on $\delta^{15}N$-NO_3^-. Nitrification tends to generate isotopically light nitrate, so a reduction in nitrification would be expected to raise $\delta^{15}N$ values. In contrast, denitrification preferentially removes ^{14}N, leaving the residual nitrate enriched in ^{15}N; thus, a decrease in denitrification would lessen this enrichment and result in lower $\delta^{15}N$ values. In the present experiment, $\delta^{15}N$-NO_3^- declined with increasing CO_2 concentration, suggesting that the balance between these two processes shifted specifically, that denitrification was more suppressed than nitrification under elevated CO_2. This isotopic response provides strong evidence that, during isotope fractionation, the product became isotopically lighter while the residual substrate became heavier, consistent with denitrification being the more strongly inhibited pathway. Therefore, the weakening of nitrification will lead to the overall positive value of $\delta^{15}N$-NO_3^- in the soil, while the weakening of denitrification will lead to the overall negative value of $\delta^{15}N$-NO_3^- in the soil. The results of this experiment show that the nitrogen isotope value gradually becomes negative with the increase of CO_2 concentration, so it can be considered that the degree of denitrification is weakened more than that of nitrification. The isotopic fractionation effect of denitrification is very strong, which is one of the main reasons for the enrichment of $\delta^{15}N$ in soil (Fang *et al.* 2015). At the same time, denitrification is an important way of nitrogen loss in terrestrial ecosystems, the loss through denitrification can reach 64% of the total NO_3^-. In conclusion, soil nitrate nitrogen isotope can further trace the soil nitrogen cycle process when CO_2 concentration increases.

4.3. *Effects of Elevated CO_2 on Nitrogen Fixation and Plant Nitrogen Utilization in the Alfalfa-Soil System on the Loess Plateau*

In the process of plant growth, NO_3^--N and NH_4^+-N are the two main mineral nitrogen sources that can be absorbed and utilized. Compared with the concentration of natural atmospheric CO_2, the contents of NO_3^--N and NH_4^+-N in alfalfa leaves increased in different degrees with the increase of CO_2 concentration. Relevant studies have shown that the increase of CO_2 concentration leads to the increase of root exudates and some non-structural carbohydrates, which increases the number and activity of soil microorganisms and may accelerate the decomposition and nitrogen mineralization of soil organic matter, and is conducive to the absorption of nitrogen by plants (Bassirirad 2000; Chowdhury *et al.* 2011). The results indicated that the increase of CO_2 concentration could promote nitrogen absorption and improve nitrogen utilisation efficiency in alfalfa. At the same time, as discussed in of the above-result section, the content of total inorganic nitrogen in the soil decreased with the increase in CO_2 concentration. Part of the reason may be due to the increased use efficiency of nitrogen-fixing plant alfalfa for nitrogen in the soil. Moreover, because the inorganic nitrogen in the soil is easily dissolved in water, and migrates with the process of soil and water loss. Therefore, reducing the total inorganic nitrogen content in the soil reduces the nitrogen that is easily lost in the soil and further enhances the nitrogen fixation in the soil (Dong *et al.* 2020). In conclusion, under the semi-arid climate conditions of the Loess Plateau, with the increase of CO_2 concentration, the nitrogen use efficiency of nitrogen fixing plant alfalfa in the soil system was enhanced, and the nitrogen fixation of soil was improved.

The experimental results showed that the $\delta^{15}N$ value of alfalfa leaves gradually increased with the increase of nitrogen content in leaves, which was consistent with the conclusions of other researchers (Martinelli *et al.* 1999; Pardo *et al.* 2006). The isotopic fractionation effect exists in plant nitrogen absorption, plants will preferentially absorb and utilize ^{15}N-depleted N and enrich the substrate ^{15}N. The key factors that restrict plant growth usually also play an important role in the process of plant isotope fractionation. For example, higher temperatures lead to more complete nitrogen assimilation and conversion processes within plants, which may reduce isotope fractionation during nitrogen assimilation and conversion, resulting in plant enrichment of ^{15}N (Amundson *et al.* 2003; Craine *et al.* 2009). Elevated CO_2 usually coincides with rising temperatures. However, the effects of CO_2 concentration and temperature on plant nitrogen isotopes need to be further improved in future studies.

4.4. *Impact of Elevated CO_2 on Soil Nitrogen Dynamics and Isotope Ratios*

The results of the experiment demonstrate significant changes in soil nitrogen content and isotope ratios under increased CO_2 concentrations, which have important implications for soil nutrient cycling and microbial processes. At 500 ppm and 700 ppm CO_2, the ammonium nitrogen content in the soil increased by 71% and 4%, respectively, while nitrate nitrogen and total dissolved nitrogen decreased significantly, with the most pronounced reductions occurring at 700 ppm CO_2. The response of soil NH_4^+-N to higher CO_2 concentration showed a clear non-linear trend with a significant rise at 500 ppm then a near-plateau at 700 ppm, which is evidence of a saturation effect in the system. The initial increase under 500 ppm can be attributed by the improved carbon levels in roots, that trigger microbial activity and enhance soil mineralization and ammonification processes of soil organic nitrogen, leading to increased NH_4^+ production. However, this stimulatory effect does not seem to persist at higher CO_2 levels (700 ppm), likely because of substrate limitation as readily mineralizable organic nitrogen is increasingly depleted. Moreover, a higher microbial biomass under elevated CO_2 level may also enhance nitrogen immobilization, whereby NH_4^+ is rapidly assimilated by microorganisms that limiting its accumulation in the soil. Furthermore, the modifications of soil moisture and oxygen availability induced by CO_2 may inhibit the nitrification process and alter microbial activity, which further limits the net NH_4^+ accumulation. All these interacting mechanisms give a reasonable explanation of the observed saturation response of NH_4^+-N at 700 ppm CO_2. The nitrogen isotope values decreased from 11.6‰ to 9.9‰ and 6.6‰ at 500 ppm and 700 ppm CO_2, respectively, while the oxygen isotope values decreased from 11.3‰ to 8.8‰ and 6.7‰ at these concentrations. These changes suggest shifts in microbial activity and nitrogen cycling processes due to elevated CO_2, with a stronger impact observed at higher CO_2 concentrations.

4.5. *Ammonium Nitrogen Increase*

The increase in ammonium nitrogen under elevated CO_2 concentrations, especially at 500 ppm, likely reflects enhanced microbial ammonification, where soil microbes convert organic nitrogen to ammonium (Ollivier *et al.* 2011). The relatively smaller increase at 700 ppm CO_2 suggests that microbial processes may become less responsive at higher CO_2 concentrations due to potential nutrient limitations or altered microbial community composition (Butterbach-Bahl *et al.* 2011).

4.6. *Decreased Nitrate and Total Dissolved Nitrogen*

The decline in total dissolved nitrogen (TDN) under high CO_2 suggests that soil nitrogen transformations have shifted beyond just a loss of inorganic nitrogen. TDN includes both inorganic forms (NO_3^--N, NH_4^+-N) and dissolved organic nitrogen (DON), so a reduction here reflects a combined response across several nitrogen pools. This could mean that mineralization of soil organic matter slowed down, resulting in less DON production or it could be that plants, growing more vigorously under elevated CO_2, took up more dissolved nitrogen to meet higher demand. Changes in microbial activity and turnover might also play a role, possibly limiting how much dissolved nitrogen gets released into the soil solution. What interesting is that this TDN decrease is a more integrated signal of both organic and inorganic nitrogen processes, unlike the drop in NO_3^--N, which mostly points to inhibited nitrification and denitrification. So overall, the decline in TDN probably reflects a broader shift in soil nitrogen availability and recycling under high CO_2 conditions. The significant decrease in nitrate nitrogen and total dissolved nitrogen at both CO_2 concentrations can be attributed to several factors. Elevated CO_2 has been shown to reduce nitrification (the conversion of ammonium to nitrate) due to reduced oxygen availability in the soil and altered microbial activity (Chung *et al.* 2007). At higher CO_2 levels, changes in soil moisture and temperature may favor denitrification, a microbial process that converts nitrate to nitrogen gases, further contributing to the reduction in nitrate and dissolved nitrogen content (He *et al.* 2014).

4.7. *Nitrogen and Oxygen Isotope Changes*

The observed decreases in nitrogen and oxygen isotope values under elevated CO_2 concentrations are consistent with enhanced ammonification and denitrification processes. Nitrogen isotope values reflect the preference of certain microbial processes for lighter nitrogen isotopes (e.g., ^14N), while oxygen isotope values are influenced by changes in soil water dynamics and microbial activity (Butterbach-Bahl *et al.* 2011; Fang *et al.* 2015). Oxygen isotopic composition of nitrate ($\delta^{18}O$) offers an additional window into nitrate formation pathways and the water-related constraints on nitrogen cycling. In this experiment, $\delta^{18}O$ values in alfalfa leaves decreased as CO_2 concentrations increased a pattern that suggests a greater contribution from newly nitrified nitrate formed in the soil. During nitrification, most oxygen atoms incorporated into nitrate come from soil water and atmospheric O_2, which together yield a lower $\delta^{18}O$ signature compared to existing nitrate pools. So, the drop we observed in leaf $\delta^{18}O$ points to a heightened reliance on nitrate produced locally through microbial nitrification. Taken alongside the $\delta^{15}N$ data, this trend indicates that elevated CO_2 not only alters microbial nitrogen transformations and plant nitrogen uptake but also reflects shifts in soil moisture and water nitrogen interactions within the soil-plant system.

4.8. *Effects of CO_2 on nitrogen cycle*

To help visualize how elevated CO_2 interacts with nitrogen cycling processes in this study, we present a conceptual schematic diagram in **Figure 7**. The figure summarizes the proposed mechanisms linking soil microbial processes, nitrogen transformations including nitrification and denitrification and plant nitrogen uptake.

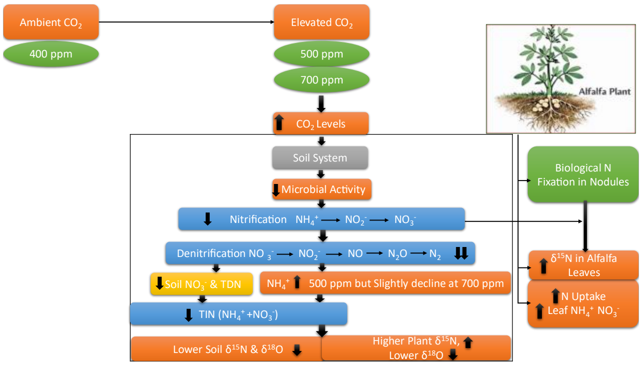


Figure 7. Conceptual framework illustrating the influence of elevated CO_2 on soil nitrogen cycling, microbial processes, plant nitrogen uptake and isotopic composition in alfalfa-soil system.

4.9. *Limitations and Future Research Directions*

Although this research provides new evidence that how the impact of high CO_2 concentration, on nitrogen cycling in alfalfa-soil system on the Loess Plateau under semi-arid environments, there are a couple of limitations that must be noted. Firstly, the soil temperature was not monitored continuously and as high CO_2 and soil warming tend to occur concomitantly in climate change the current design can not isolate their respective effects on the processes of transformation of nitrogen. Indirectly, microbial responses were also determined by analysis of nitrogen pools and isotopic signatures, rather than being measured directly through determination by community composition or functional gene marker (e.g. *amoA*, *nirS, nirK*). Additionally, the experiment covered only one growing season in one place, which, of course, restricts the extent to which the results can be generalized in space and time. In the future, the combination of continuous soil temperature data with multi-site, multi-year experiments and the incorporation of molecular tools to examine important functional genes would assist in elucidating the interactions between CO^2, temperature and microbial processes to influence nitrogen cycling in semi-arid environments.

5. Conclusion

In the semi-arid environment of the Loess Plateau, the increase in atmospheric CO_2 significantly changed the relationships between nitrogen in the alfalfa-soil system. The CO_2 increased soil NH_4^+-N, while NO_3-N and total dissolved nitrogen decreased indicating a change in the manner in which nitrogen transformations were made. This was supported by isotopic evidence, as $\delta^{15}N\text{-}NO_3^-$ decreased with an increase in CO_2 concentration, indicating that both the process of nitrification and denitrification were inhibited, although denitrification was more severely impacted. In the meantime, there was an increase in nitrogen concentrations in the alfalfa leaves due to high CO_2 which is an indication of increased nitrogen uptake and improved nitrogen use efficiency in alfalfa. Probably that augmented uptake by plants decreased the soil inorganic nitrogen pool and possibly one of the factors that kept more nitrogen in the soil-plant system. Combined, we discover that at high CO_2 levels a noticeable imprint can be made on soil nitrogen cycling, microbial processes and plant nitrogen use in semi-arid ecosystems. Stable nitrogen isotopes that are not volatile became a helpful tool to trace those transformations, which provided a more accurate picture of how the nitrogen dynamics may act in the future under the conditions of the changing climatescenarios. Such insights are important not only in the context of forecasting ecosystem reactions, but also in the context of sustainable land management as CO_2 levels continue to increase.

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Ethical approval

Not applicable

Consent to participate

Not applicable

Consent to publish

Not applicable

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions:

All authors contributed to the study conception and design. Data preparation, sample collection, and data analysis were performed by Tauhid Khan.

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