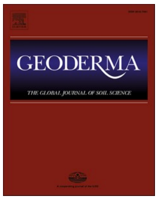
Geoderma 452 (2024) 117106

Contents lists available at ScienceDirect

Geoderma

journal homepage: www.elsevier.com/locate/geoderma



Elucidating the role of earthworms on the fate of fertilizer N with synthetic and organic fertilizer application

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ARTICLE INFO

Handling Editor: Y. Capowiez

Keywords:
Earthworms
N transformations
Pot experiment
Soil-plant system
N isotope

ABSTRACT

Earthworms' activities not only increase soil nitrogen (N) uptake by crops but also lead to N losses to environment. However, it remains unclear whether earthworms' impact on the fate of fertilizer N differs based on the type of fertilizer application. Therefore, the present pot experiment examined the transformation and fate of two types of ^{15}N-labeled fertilizer (synthetic fertilizer urea and organic fertilizer compost applied at rate of 400 mg N/pot) with and without earthworms (*Amynthas corticis*) in a soil-lettuce system over three seasons of cultivation. Results showed that earthworms increased the fresh biomass of lettuce in all three seasons, regardless of the type of fertilizer used. However, the effect of earthworms on fertilizer N uptake varied depending on the type of fertilizer. With earthworms present, lettuce took up an additional 20.97 mg/pot of synthetic fertilizer N in the first season, which sharply decreased to 2.72 mg/pot and 4.63 mg/pot in the second and third seasons, respectively. In contrast, the uptake of organic fertilizer N by lettuce increased by 10.08–11.24 mg/pot throughout the entire experiment when earthworms were present. The presence of earthworms increased the percentage of synthetic fertilizer N lost to the environment by 0.8 %, due to increased N leaching, N_2O emission, NH_3 volatilization, etc. In contrast, earthworms decreased the percentage of organic fertilizer N lost to the environment by 1.9 %, primarily through reduced NH_3 volatilization, etc. This study underscores the pivotal role of earthworms in modulating fertilizer N dynamics, with organic fertilizer offering superior ecosystem services compared to synthetic fertilizer. Given that only one earthworm species was studied and nearly half of the organic fertilizer remained in the soil, future long-term experiments incorporating diverse earthworm species and changes in the soil's native N pool are essential to fully understand the role of earthworms in agro-ecosystem N cycling.

1. Introduction

The application of nitrogen (N) fertilizer has significantly supported the rapid increase in crop production over the past few decades. However, despite significantly supporting this increase, only a small proportion of the applied N is incorporated into crops. Substantial amounts of the applied N either remaining in the soil profile or being lost to the environment through runoff, leaching, and gas emissions (Ladha et al., 2016). Consequently, the fate of fertilizer N in agro-ecosystems has garnered considerable attention due to its direct impact on fertilizer efficiency and environmental concerns.

Fertilizers are broadly classified into two main types: synthetic fertilizers and organic fertilizers. The type of fertilizer used influences the fate of N after application. Urea is the predominant synthetic N fertilizer in agricultural systems, accounting for more than 50 % of global N fertilizer applications (Wiesler, 1998). Urea is a readily available N source for plants, entering either through direct uptake or by hydrolysis into ammonia in the soil facilitated by the enzyme urease (Merigout et al., 2008). However, due to the ease of solubility and the rapid hydrolysis process, plants are unable to access 40–70 % of the N released from urea (Beig et al., 2020). The hydrolyzed ammonium ion from urea can further converted into ammonia gas (i.e., NH_3) and other reactive

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https://doi.org/10.1016/j.geoderma.2024.117106
Received 13 August 2024; Received in revised form 9 November 2024; Accepted 11 November 2024

nitrogen compounds (e.g., nitrate, NO and N_2O), which may volatilize into the atmosphere or leach into surface and groundwater, contributing to environmental issues (Wang et al., 2018). In contrast, organic fertilizers, such as manure compost, are less soluble. The N in organic fertilizer is gradually available to the plant and thus showed a long-lasting effect to plants (Geng et al., 2019). Duan et al. (2016) quantified the impact of various fertilization regimes on soil N storage and loss across different spatiotemporal scales, demonstrating a significant increase in soil N storage with the lowest N loss under manure incorporation, whereas chemical fertilization treatments posed a higher risk of N loss.

Earthworms are key components of the soil fauna and play a crucial role in driving soil N dynamics (De Vries et al., 2013). Their presence accelerates organic N mineralization, contributing 5–25 % of plant N requirements and significantly increasing crop biomass by 21 %, according to a *meta*-analysis (Van Groenigen et al., 2014). However, excessive mineral N released from organic matter can lead to N losses through denitrification and leaching. Earthworms have been reported to increase soil N_2O emissions by an average of 42 % (Lubbers et al., 2013), and increase N leaching from soil due to the preferential flow pathways created by earthworm burrows (Domínguez et al., 2004). In this way, earthworms simultaneously act as both a service (enhancing N utilization by plants) and a disservice (causing N losses to the environment) by influencing soil N dynamics (Wu et al., 2015). Several studies have focused on the impact of earthworms on fertilizer N dynamics. For example, with urea application, earthworms facilitated the transfer of fertilizer N to lettuce without significantly increasing fertilizer N loss to the environment (Na et al., 2022b). With straw return, earthworms stimulate short-term mineralization of native soil N, making it available for uptake by lettuce and increasing the risk of loss as N_2O, while temporarily stabilizing straw N in the soil (Na et al., 2022a). However, these studies were conducted separately, and the short duration (one season) of the experiments may not be sufficient to comprehensively evaluate the effects of earthworms on fertilizer N dynamics, as fertilizer N remaining in the soil may still be utilized by plants in the future. This is particularly relevant for organic fertilizers, which require a longer period for mineralization and release N more slowly and steadily compared to synthetic fertilizers (Sradnick and Feller, 2020). Therefore, further research is needed to fully understand the complex interactions between earthworms and fertilizer N dynamics.

Fertilization represents the primary source of nitrogen in agricultural ecosystems. The objective of the present study is to track the fate of fertilizer nitrogen (N) in a crop-soil system, particularly focusing on the influence of earthworm activity. Given the pivotal role of earthworms in soil N dynamics, this study aims to offer a comprehensive assessment of the ecosystem services and disservices associated with earthworms. Additionally, it seeks to provide insights into how soil biota, particularly earthworms, influence N cycling under different types of fertilizer applications. We hypothesized that (1) earthworm activities would stimulate N cycling, potentially increasing both N uptake by plants and N losses to the environment concurrently, and (2) the magnitude of this effect may vary depending on the type of fertilizer. To test these hypotheses, we conducted a three-season cultivation experiment in which synthetic (urea) and organic (compost) fertilizers were applied, to examine the amount of fertilizer N retained in the bulk soil, taken up by plants, and lost to the environment in a soil-lettuce system with and without earthworms.

2. Material and methods

2.1. Soil, earthworms, fertilizer and plants

Soil was collected from the top layer (0–20 cm) of a vegetable field located in Dangyang, Hubei Province, China (111°48′E, 30°39′N). The management practices of this field for controlling pest, disease and weeds complied with local practices for green food production. Fresh soil was sieved to pass 5 mm sieve, and then moistened with distilled water to achieve 65 % of water filled pore space for seven days pre-incubation. The soil was classified as yellow–brown earth with a silty texture, consisting of 12.86 % sand, 47.51 % silt, and 39.63 % clay. It had a pH of 6.13 when measured in a 1:5 soil-to-water ratio. The soil cation exchange capacity (CEC) was 11.24 cmol/kg. The soil organic matter (SOM) content and total nitrogen (TN) were 17.31 and 1.42 g/kg, respectively, with a C/N at 7.01 and a $\delta^{15}N$ value of 4.30 ‰.

The earthworm density in the vegetable field from which the soil was collected was 51 individual/m^2. The dominant adult earthworm species, *Amynthas corticis*, were collected using a hand-sorting method. *A. corticis* is a cosmopolitan epigeic species known for its great adaptability and ability to reproduce through polyploid or parthenogenetic means, making it widespread across the globe (Wang et al., 2021). The earthworms were placed on wet filter paper for 24 h to clear their guts, rinsed with sterilized distilled water, and then weighed before use. The mean fresh weight of the earthworms was 3.72 g, with a N content of 82.60 g/kg and a $\delta^{15}N$ value of 9.50 ‰ in their whole-body tissue.

Urea and cow dung compost were selected as synthetic and organic fertilizers, respectively. The ^{15}N-urea (with 46.60 % of N content and 26682 ‰ of $\delta^{15}N$) were purchased from Shanghai ZZBIO Co., Ltd (Shanghai, China). The ^{15}N-labeled rice straw was produced by a ^{15}N pulse labeling experiment conducted in a greenhouse. A cow was then fed with the ^{15}N-labelled straw for two weeks to produce ^{15}N-labelled manure. Then, ^{15}N-labelled manure and straw were mixed (70 % manure + 30 % straw) and composted for 20 days to create ^{15}N-labelled compost. The obtained ^{15}N-labeled compost was air-dried and subsequently ground <3 mm with a ball mill grinder. The compost contained 14.31 g of total N/kg, giving a C/N ratio of 27.41, with a $\delta^{15}N$ value of 45028 ‰.

Previous studies have demonstrated that only a small proportion of applied fertilizer N is utilized by plants in the current growing season, with a substantial amount of fertilizer N remaining in the soil for potential use in subsequent seasons (Cook and Davis, 1957). Consequently, the present incubation experiment included a total of three seasons of plant cultivation (Fig. 1). Before each planting season, lettuce (*Lactuca sativa L. var. longifolia Lam.*) seedlings were grown in culture substrate to four-leaf stage. Individuals of similar size were then selected for the experiment. The average fresh weight, tissue N content, and δ15N signature of the lettuce used for each season's transplantation are shown in Table S1.

2.2. Experimental design

The microcosm experiment was designed with four treatments: (1) synthetic fertilizer (urea) application with and without earthworms (S + E and S-E), and (2) organic fertilizer (compost) application with and without earthworms (O + E and O-E). Treatments with no nitrogen fertilizer were also included, but these were used solely for the calculation of ^{15}N distribution. Consequently, data from the treatments without nitrogen fertilizer are not presented in this study.

The experimental units consisted of plastic pots (20 cm in diameter and 15 cm in height) with several holes (0.3 cm in diameter) at the bottom to allow excess water (leachate) to drain. Fifteen pots were prepared for each treatment, and all pots were randomly placed in the experimental facility. Fresh soil (equivalent to 2.5 kg of dry soil) was added to each pot, thoroughly mixed with different fertilizer at a dose of 160 mg N/kg dry soil (equivalent to 400 mg of ^{15}N in each pot), and packed to a bulk density of 1.2 g/cm^3, which is comparable to field soil bulk density. Two earthworms were inoculated into the pots corresponding to the treatments with earthworm presence. A higher earthworm density than field was used because (1) earthworm populations up to 277 individuals/m^2 have been reported in Chinese agroecosystems, and (2) a relatively higher population in the soil microcosms allowed for easier observation of the earthworms' effects (Na et al., 2022a). Then, a lettuce seedling was transplanted into each pot for the first cultivation season (Fig. 1). All pots were incubated at ambient temperature in a rain

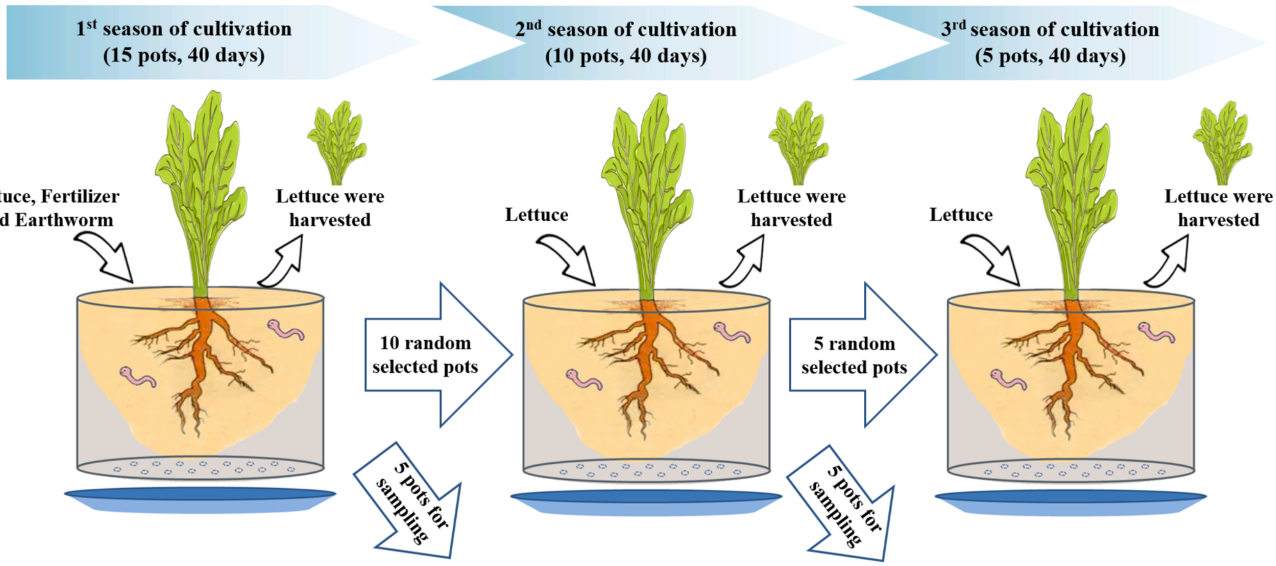

Fig. 1. Pot Experiment for Tracking Fertilizer Nitrogen Transformations in a Soil-Lettuce System Fifteen pots were allocated for each treatment. After each season of harvesting, five randomly selected pots were used for soil and earthworm sampling, while the remaining pots were carried over for the next season of cultivation. No additional fertilizer or earthworms were added; instead, new lettuce seedlings were transplanted for the second and third seasons.

shelter for 40 days until the first harvest. After harvesting, five randomly selected pots per treatment were used for destructive soil and earthworm sampling, while the remaining ten pots were used for the second cultivation season. No additional fertilizer or new earthworms were added during the subsequent incubation periods, but a new lettuce seedling was transplanted at the beginning of each season. Plants were harvested at the end of the second season, with five randomly selected pots per treatment were used for soil and earthworm sampling, and the remaining five pots were used for the third cultivation season.

Each cultivation season lasted 40 days, during which distilled water was added to each pot based on the actual rainfall recorded at the field site. In the first season, 312 ml of distilled water was added on day 19 and 133 ml on day 28. In the second season, 157 ml of distilled water was added on day 13, 284 ml on day 15, and 174 ml on day 32. In the third season, 177 ml of distilled water was added on day 7 and 162 ml on day 28.

2.3. *Sampling and analysis*

During each season of cultivation, N_2O emission and NH_3 volatilization were measured daily until harvest. N_2O emissions were measured using the closed chamber method, with gas samples analyzed by a gas chromatograph (GC-7890A, Agilent Technologies, USA) equipped with a flame ionization detector. The ^{15}N enrichment of N_2O was analyzed by isotope-ratio mass spectrometer (MAT253, Finnigan, USA) coupled with a pre-concentration unit·NH_3 volatilization was determined by venting method (Wang et al., 2002). Specifically, PVC tubes containing two foams soaked in phosphoglycerol were inserted into the soil. The bottom foam disc absorbed NH_3–N volatilized from the soil, while the upper disc buffered the ambient NH_3 concentration. The NH_3–N absorbed in the bottom foams was extracted immediately with KCl solution, and the concentration of NH_4^+–N in the extract was measured using a continuous segmented flow analyzer (AA3, SEAL Analytical, Germany). The ^{15}N enrichment in volatilized NH_3 was determined using an isotope-ratio mass spectrometer (MAT253, Finnigan, USA) with freeze-dried extract. Cumulative gas (N_2O and NH_3) emission were calculated by linear interpolation between measurement days (Na et al., 2022a).

Drainage water was collected from the disc below each pot, and the volume of leachate was recorded. The total N content in the leachate was measured using a spectrophotometric method, while the ^{15}N enrichment was analyzed with an isotope-ratio mass spectrometer (MAT253, Finnigan, USA) using freeze-dried leachate.

At the end of each season, whole lettuce plants were collected. After weighing the fresh biomass, the plants were oven-dried. Earthworms were carefully separated from the soil, then rinsed and allowed to void their guts for 24 h before their fresh biomass was measured. The earthworms were then freeze-dried. Fresh soil was either sieved through a 2 mm mesh for chemical and microbial analyses or air-dried for ^{15}N analysis. The dried plant, earthworm, and soil samples were ground and analyzed for total N and ^{15}N using an isotope-ratio mass spectrometer (MAT253, Finnigan, USA) coupled with an elemental analyzer (Flashsmart CN, Thermo Fisher, Germany).

Fresh soil was extracted by KCl solution and then analyzed for soil inorganic N (Inorg-N) content by continuous flow analyzer (AA3, SEAL Analytical, Germany). The KCl-extracted solution was reduced by Devarda's alloy method and then freeze-dried for Inorg-N isotope analysis by mass spectrometry (MAT253, Finnigan, USA). Soil microbial biomass N (MBN) was determined by chloroform fumigation extraction method. Briefly, two fresh soil subsamples were prepared: one was fumigated with $CHCl_3$ and the other was left unfumigated. The N content of both fumigated and unfumigated samples was extracted with K_2SO_4 solution and determined by elemental analyzer (Flashsmart CN, Thermo Fisher, Germany). MBN was calculated as the difference in N content between the fumigated and unfumigated sample. The ^{15}N abundance of fumigated and unfumigated extracts was determined as above described for inorganic ^{15}N analysis.

2.4. *Calculations and statistical analysis*

The percentage of N derived from fertilizer (Ndff, %) in the samples was calculated using the follow equation (Lukas et al., 2013):

$$\mathrm{Ndff}(\%) = \left[\left(\delta^{15}\mathrm{N}_{\mathrm{sample}} - \delta^{15}\mathrm{N}_{\mathrm{control}}\right) / \left(\delta^{15}\mathrm{N}_{\mathrm{fertilizer}} - \delta^{15}\mathrm{N}_{\mathrm{control}}\right)\right] \times 100$$

where $\delta^{15}N_{sample}$ (‰) represents the $\delta^{15}N$ value of each sample (e.g. soil, plant, earthworm, gas and leachate) in the treatment with fertilizer, $\delta^{15}N_{control}$ (‰) is the $\delta^{15}N$ value of corresponding samples in the treatment without fertilizer, and $\delta^{15}N_{fertilizer}$ (‰) is the initial $\delta^{15}N$ value of applied fertilizer.

At each end of season, fertilizer N assimilated by plant or earthworm was calculated as:

$$\text{Fertilizer N assimilated (mg/pot)} = \text{Dry biomass (g/pot)} \times \text{N content (g/kg)} \times \text{Ndff (\%)}/100$$

where Dry biomass (g/pot) represents the dry biomass of plant or earthworm, N content (g/kg) is the total N content of corresponding samples.

Soil native N assimilated by plant or earthworm was calculated as:

$$\text{Soil N assimilated (mg/pot)} = \text{Dry biomass (mg/pot)} \times \text{N content (g/kg)} - \text{Fertilizer N assimilated (mg/pot)} - \text{Initial N content (mg/pot)}$$

where initial N content is the total N content in the plant or earthworm before transplantation.

As earthworm accumulated N will release to soil again in future by their metabolic products (casts, urine and mucus) and dead tissues (Blouin et al., 2013), we consider the fertilizer N in earthworm body tissues as part of the soil N pool. Thus, the residual fertilizer N in soil was calculated as:

$$\text{Residual fertilizer N in soil (g/pot)} = \text{Earthworm accumulated N fertilizer (g/pot)} + [\text{Dry weight}_{\text{soil}}\text{(g/pot)} \times \text{N conten}_{\text{soil}}\text{(g/kg)} \times \text{Ndff}_{\text{soil}}\text{(\%)} \times 1000$$

N is one of the most vulnerable macronutrients to environmental loss, by NH_3 volatilization, soil denitrification (NO_x flux), leaching, and surface runoff, etc. (Mahmud et al., 2021). Tracking all forms of N loss is challenging. Therefore, potential N losses in this study were estimated using a mass balance calculation method: the difference between total amount of applied N and amount of recovered N from plants and soil. This method encompasses N losses from both the current and previous cultivation seasons.

All data met the assumptions of normality. Treatments were compared by one-way analysis of variance (ANOVA) and least significant difference (LSD) at the 5 % level. Statistical analyses were done with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Plants biomass and nitrogen uptake

Overall, the presence of earthworms significantly increased lettuce fresh biomass across all three seasons of cultivation, regardless of the type of fertilizer used (Fig. 2A). However, this effect was more pronounced with organic fertilizer ($P < 0.01$) compared to synthetic fertilizer ($P < 0.05$). With synthetic fertilizer, lettuce fresh biomass significantly decreased ($P < 0.05$) in the second and third seasons compared to the first season. In contrast, there was no significant change in lettuce fresh biomass between different seasons when organic fertilizer was used.

In the synthetic fertilizer treatment, fertilizer N was the primary nitrogen source for lettuce during the first season of cultivation, accounting for 74–78 % of the plant's nitrogen content. However, this

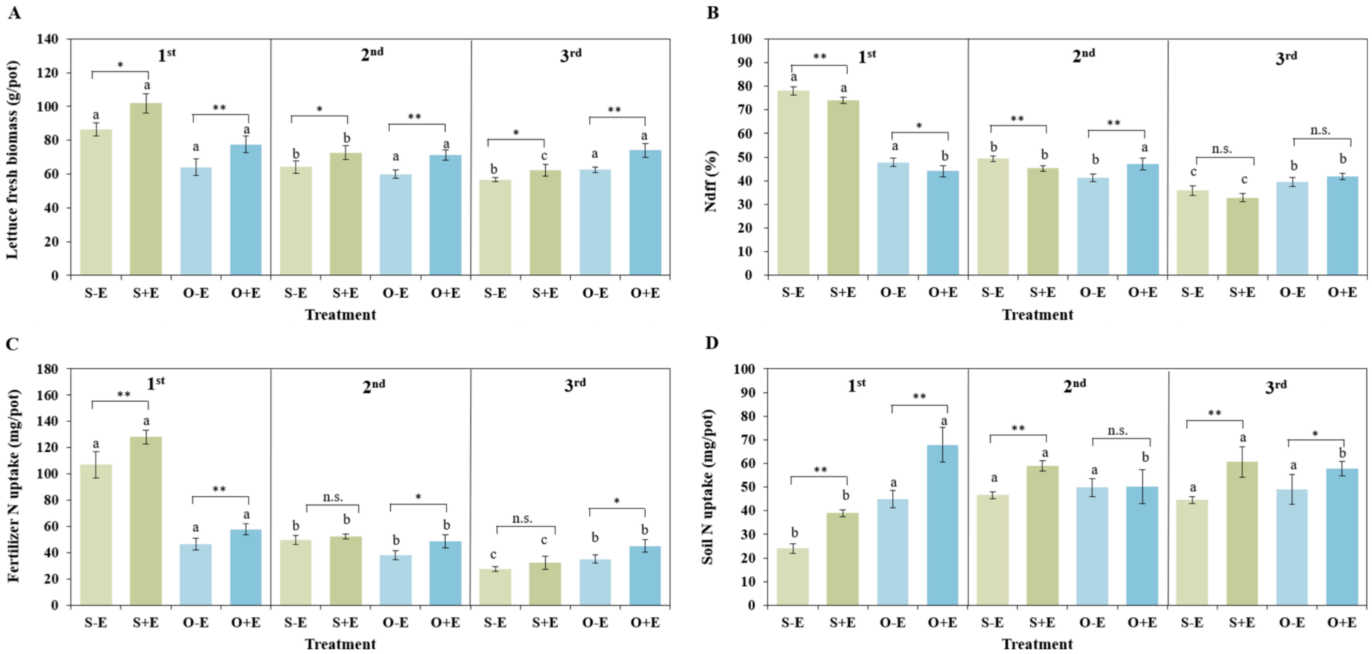

Fig. 2. Lettuce Biomass and Nitrogen Uptake (A) shows lettuce biomass. (B) displays the percentage of total nitrogen in lettuce derived from fertilizer. (C) and (D) represent the amounts of fertilizer nitrogen and soil native nitrogen uptake by lettuce, respectively. 1st, 2nd, and 3rd denote the first, second, and third seasons of cultivation. Lowercase letters indicate significant differences ($P < 0.05$) between different seasons for the same treatment. Asterisks above black lines denote significant differences between earthworm presence and absence for the same type of fertilizer in each season (** significant at $P < 0.01$; * significant at $P < 0.05$; n.s. not significant). S + E and S-E indicate urea application with and without earthworms, respectively. O + E and O-E indicate compost application with and without earthworms, respectively.

proportion dropped sharply to less than 50 % in the second and third seasons (Fig. 2B). In contrast, in the organic fertilizer treatment, the percentage of plant's nitrogen derived from the fertilizer remained consistently between 39.5 to 47.8 % throughout the entire experiment. Earthworms significantly increased ($P < 0.01$) the uptake of synthetic fertilizer N by lettuce during the first season. However, this effect was not significant in the subsequent seasons (Fig. 2C). In contrast, earthworms significantly enhanced ($P < 0.01$ in the first season and $P < 0.05$ in the subsequent seasons) the uptake of organic fertilizer N by lettuce throughout the entire experiment. With the addition of synthetic fertilizer, earthworms significantly increased ($P < 0.01$) the uptake of soil native N by lettuce throughout the entire experiment (Fig. 2D). In contrast, with organic fertilizer, the significant effect of earthworms on enhancing soil native N uptake by lettuce was observed only in the first ($P < 0.01$) and third ($P < 0.05$) seasons.

3.2. Earthworms biomass and nitrogen content

Compared to the beginning of the experiment, earthworms' fresh biomass increased by approximately 18 % with synthetic fertilizer and by 23 % with organic fertilizer by the end of the first season (Fig. 3A). However, a decline in earthworm biomass was observed in the subsequent seasons, particularly during the third season, which showed a significant decrease ($P < 0.05$) compared to the first season. Although there was no significant difference in earthworm biomass between different fertilizer treatments, a notably higher percentage of fertilizer-derived nitrogen was found in earthworm tissue with organic fertilizer application during the second season (Fig. 3B).

During the first and second seasons, earthworms assimilated significantly ($P < 0.05$ and $P < 0.01$, respectively) more fertilizer nitrogen with organic fertilizer than with synthetic fertilizer (Fig. 3C). Regardless of the type of fertilizer used, the amount of fertilizer nitrogen assimilated by earthworms significantly decreased ($P < 0.05$) across the seasons. The pattern of soil native nitrogen assimilation by earthworms was similar to that of fertilizer nitrogen assimilation (Fig. 3D).

3.3. Soil total nitrogen, microbial biomass nitrogen and inorganic nitrogen content

Earthworms significantly ($P < 0.05$) reduced soil TN content throughout the entire experiment, regardless of the type of fertilizer applied (Table 1). Soil $T^{15}N$ content was significantly ($P < 0.05$) reduced by the presence of earthworms, except in the first season with organic fertilizer application. In addition, as cultivation progressed, the TN and $T^{15}N$ content in the soil consistently decreased.

Earthworms increased soil MBN content in all treatments, but a significant difference ($P < 0.01$) between the presence and absence of earthworms was only observed in the first season with organic fertilizer application (Table 1). Earthworms significantly ($P < 0.01$ for the first and second seasons, $P < 0.05$ for the third season) increased $MB^{15}N$ content throughout the experiment when organic fertilizer was applied, but had a minor effect on increasing $MB^{15}N$ content with synthetic fertilizer.

Earthworms increased soil Inorg-N content throughout the entire experiment, regardless of the type of fertilizer applied. However, a significant difference between the presence and absence of earthworms was only observed in the first and third seasons (Table 1). The response of soil Inorg-^{15}N content to earthworms varied depending on the type of

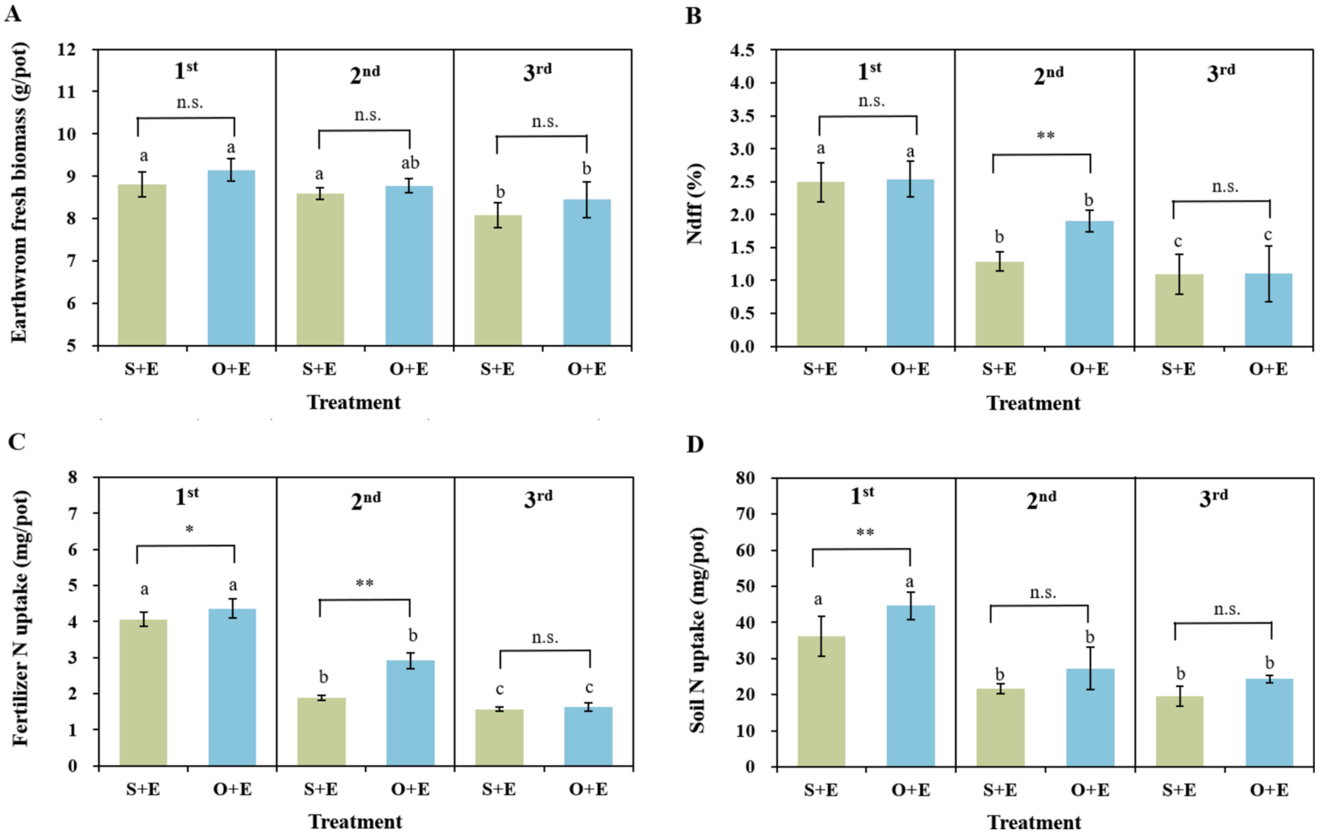

Fig. 3. Earthworm Biomass and Nitrogen Uptake (A) shows earthworm biomass. (B) displays the percentage of total nitrogen in earthworms derived from fertilizer. (C) and (D) represent the amounts of fertilizer nitrogen and soil native nitrogen uptake by earthworms, respectively. 1st, 2nd, and 3rd denote the first, second, and third seasons of cultivation. Lowercase letters indicate significant differences ($P < 0.05$) between different seasons for the same treatment. Asterisks above black lines denote significant differences between different types of fertilizer in each season (** significant at $P < 0.01$; * significant at $P < 0.05$; n.s. not significant). S + E and S-E indicate urea application with and without earthworms, respectively. O + E and O-E indicate compost application with and without earthworms, respectively.

Table 1
Soil total nitrogen, microbial biomass nitrogen and inorganic nitrogen content in different treatment.

Seasons	Treatment	Total nitrogen (g N/kg)	^{15}N total nitrogen (mg N/pot)	Microbial biomass nitrogen (mg N/kg)	^{15}N microbial biomass nitrogen (mg N/pot)	Inorganic nitrogen (mg N/kg)	^{15}N inorganic nitrogen (mg N/pot)
1st	S-E	1.41 ± 0.01 *A	236.39 ± 5.78 **A	62.6 ± 1.90 A	37.58 ± 55.44 A	106.36 ± 4.52 **A	147.48 ± 8.75 **A
	S+E	1.37 ± 0.02 *A	202.68 ± 3.37 **A	74.37 ± 4.40 A	41.21 ± 3.88 A	139.10 ± 9.10 **A	111.31 ± 8.04 **A
	O-E	1.40 ± 0.03 *A	321.91 ± 8.36 A	80.73 ± 7.63 **A	45.96 ± 2.10 **A	35.93 ± 3.31 **A	33.24 ± 4.81 **A
	O+E	1.35 ± 0.03 *A	312.59 ± 6.71 A	106.06 ± 11.54 **A	76.00 ± 8.31 **A	91.31 ± 8.44 **A	59.10 ± 7.04 **A
2nd	S-E	1.34 ± 0.01 **B	156.90 ± 7.19 **B	35.60 ± 2,78 B	21.35 ± 2.20 B	27.69 ± 3.10 B	31.09 ± 3.85 B
	S+E	1.31 ± 0.01 **B	127.45 ± 4.58 **B	36.98 ± 2.06 B	22.32 ± 0.96 B	29.69 ± 2.69 B	29.06 ± 4.07 B
	O-E	1.34 ± 0.01 *B	267.03 ± 5.61 *B	39.29 ± 2.30 B	25.65 ± 0.91 **B	15.29 ± 1.87 B	24.13 ± 3.09 B
	O+E	1.31 ± 0.01 *B	253.55 ± 8.27 *B	43.58 ± 4.80 B	31.32 ± 2.19 **B	18.49 ± 1.27 B	28.72 ± 2.53 B
3rd	S-E	1.31 ± 0.01 **C	121.44 ± 5.14 **C	26.50 ± 1.99 C	17.46 ± 1.08 C	29.14 ± 2.45 *B	35.53 ± 3.89 B
	S+E	1.27 ± 0.01 **C	88.52 ± 4.28 **C	29.10 ± 2.18 C	18.56 ± 1.01 C	35.27 ± 1.50 *B	34.56 ± 3.28 B
	O-E	1.30 ± 0.02 *C	220.67 ± 2.45 **C	39.83 ± 2.37 B	21.22 ± 0.83 *B	19.93 ± 1.42 **B	28.92 ± 2.52 B
	O+E	1.27 ± 0.01 *C	195.23 ± 10.37 **C	42.46 ± 2.08 B	23.79 ± 1.55 *C	24.14 ± 2.45 **B	34.53 ± 3.90 B

Values followed by asterisks indicate significant differences between earthworm presence and absence for the same type of fertilizer in each season (** significant at $P < 0.01$; * significant at $P < 0.05$). For the same treatment, values within the same column followed by the same uppercase letter are not significantly different ($P > 0.05$) between different seasons. S + E and S-E indicate urea application with and without earthworms, O + E and O-E indicate compost application with and without earthworms.

fertilizer. Overall, earthworms increased soil Inorg-^{15}N content with organic fertilizer application, but decreased Inorg-^{15}N content with synthetic fertilizer, showing a significant ($P < 0.01$) difference in the first season.

3.4. N_2O, NH_3 and leaching losses

Regardless of the type of fertilizer applied, earthworms significantly increased total cumulative N_2O emissions and soil native N-derived N_2O emissions throughout the entire experiment (Fig. 4A and E). Earthworms did not influence N_2O emissions derived from organic fertilizer (Fig. 4C). However, they significantly increased ($P < 0.01$) N_2O emissions derived from synthetic fertilizer in the first season.

Synthetic fertilizer application resulted in high total cumulative NH_3 volatilization (1050–1109 mg/m^2) during the first season, whereas organic fertilizer led to relatively lower cumulative NH_3 volatilization throughout the experiment (Fig. 4B). Earthworms did not significantly influence total cumulative NH_3 volatilization. However, their presence significantly increased ($P < 0.01$) NH_3 volatilization derived from fertilizer when synthetic fertilizer was applied, and significantly increased ($P < 0.05$) NH_3 volatilization derived from soil native N when organic fertilizer was applied (Fig. 4D and F).

With the addition of synthetic fertilizer, the total leaching volume was not significantly affected by earthworms (Fig. 5A). However, in the second season, earthworms significantly increased ($P < 0.05$) the total leaching volume when organic fertilizer was used. Throughout the experiment, earthworms did not significantly affect the total N leaching from either fertilizer N or soil native N (Fig. 5 B, C and D).

3.5. The fate of fertilizer nitrogen

Overall, earthworms increased the amount of fertilizer N uptake by plants in all three seasons, regardless of the type of fertilizer (Fig. 6A). However, the extent of this effect varied with the type of fertilizer. In the presence of earthworms, lettuce absorbed an additional 20.97 mg/pot of synthetic fertilizer nitrogen in the first season, representing a 19.58 % increase compared to when earthworms were absent. However, this enhancement sharply declined to 2.72 mg/pot and 4.63 mg/pot in the second and third seasons, respectively, which translated to a 15.11 % and 15.35 % increase compared to earthworms' absence. On the other hand, when earthworms were present, lettuce's uptake of organic fertilizer nitrogen increased by 10.08 to 11.24 mg/pot throughout the experiment, marking a 24.09 % to 26.49 % increase compared to conditions without earthworms.

Earthworms had differing effects on the potential loss of synthetic and organic fertilizer nitrogen to the environment (Fig. 6B). Potential N losses in this study were estimated using a mass balance calculation method, which encompassed N losses from both the current and previous cultivation seasons. Specifically, earthworms increased the loss of synthetic fertilizer N to the environment throughout the experiment, but decreased the loss of organic fertilizer N to the environment.

The total ^{15}N recovery rates are 86.63–89.09 % at the end of incubation. Introduction of earthworms significantly boosted the percentage of nitrogen from both synthetic and organic fertilizers utilized by plants. Specifically, the utilization rate of synthetic fertilizer nitrogen increased from 46.1 % to 53.2 %, while for organic fertilizer nitrogen, it rose from 30.0 % to 37.9 % (Fig. 6C). However, despite this enhancement, a notable proportion of nitrogen was still lost to the environment: 23.5–24.3 % for synthetic fertilizer nitrogen and 12.9–14.8 % for organic fertilizer nitrogen. Earthworms' presence exacerbated the loss of synthetic fertilizer nitrogen to the environment, primarily through increased leaching, N_2O emissions, NH_3 volatilization, and other forms of nitrogen loss. Conversely, earthworms helped mitigate the loss of organic fertilizer nitrogen to the environment, mainly by reducing NH_3 volatilization and other forms of loss. There were still 22.5–30.4 % of synthetic fertilizer N and 49.2–55.2 % of organic fertilizer N remaining in the soil, which can be utilized by plants in subsequent cultivation.

4. Discussions

4.1. Earthworms exhibited a more pronounced effect on enhancing the uptake of nitrogen from organic fertilizer compared to synthetic fertilizer

The results of this study demonstrated that the presence of earthworms significantly increased plant biomass throughout the experiment, regardless of the type of fertilizer used. This finding is consistent with previous research, which reported an average 23 % increase in aboveground plant biomass due to earthworms (Van Groenigen et al., 2014).

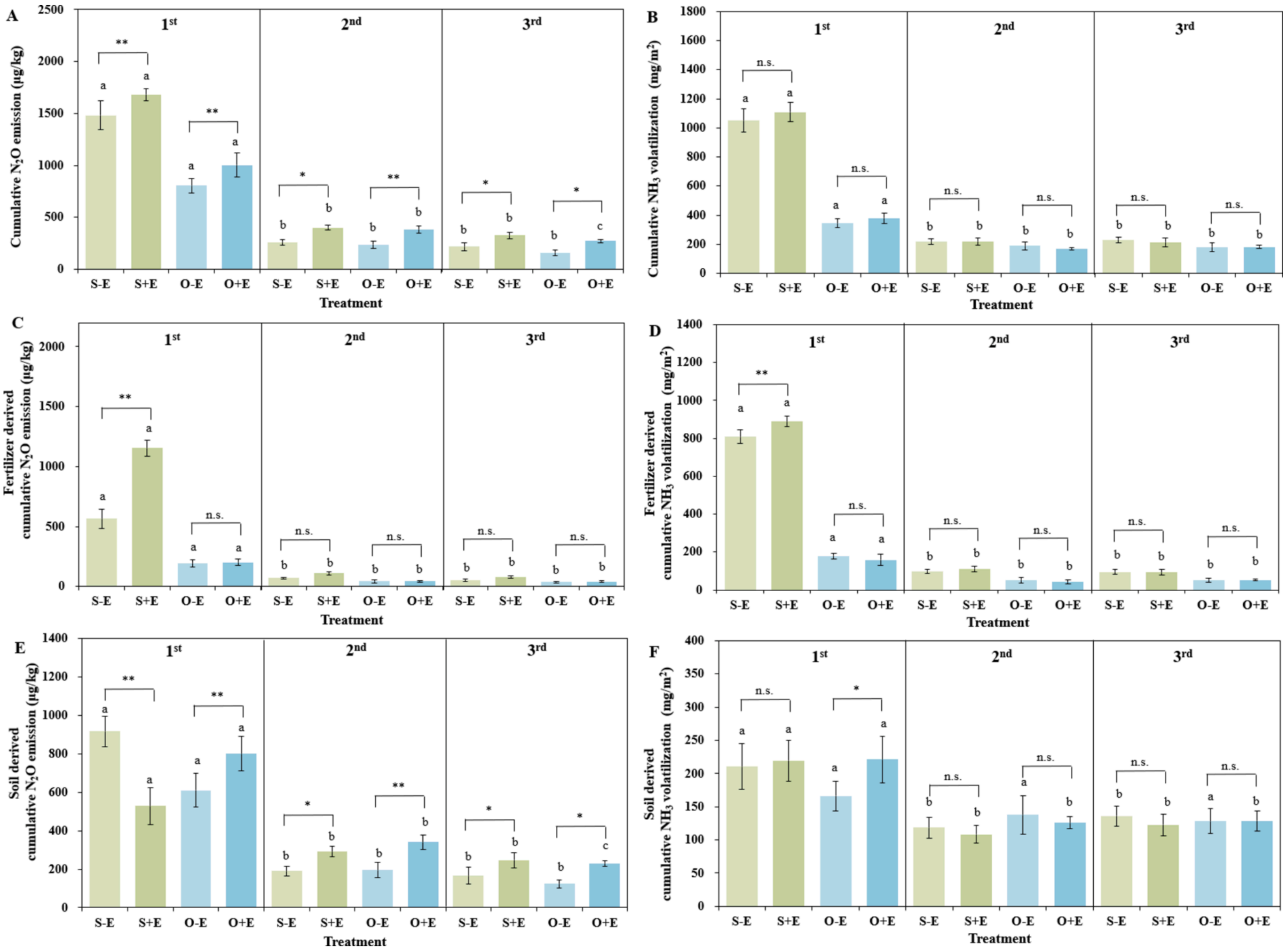

Fig. 4. Cumulative N_2O Emission and NH_3 Volatilization from Different Treatments (A), (C) and (E) shows total, fertilizer derived and soil derived cumulative N_2O emission, respectively. (B), (D) and (F) shows total, fertilizer derived and soil derived NH_3 volatilization, respectively. 1st, 2nd, and 3rd denote the first, second, and third seasons of cultivation, respectively. Lowercase letters above the columns indicate significant differences ($P < 0.05$) between different seasons for the same treatment. Asterisks above black lines denote significant differences between earthworm presence and absence for the same fertilizer addition in each season (** significant at $P < 0.01$; * significant at $P < 0.05$; n.s. not significant). S + E and S-E indicate urea application with and without earthworms, respectively. O + E and O-E indicate compost application with and without earthworms, respectively.

Increased N mineralization from organic matter, including both soil and organic fertilizers, is considered the primary mechanism by which earthworms enhance plant growth (Postma-Blaauw et al., 2006). Consequently, studies utilizing organic fertilizers observed a more substantial effect on plant growth (+34 %) compared to those using synthetic fertilizers (+10 %) (Van Groenigen et al., 2014). The present study corroborates these findings and further clarifies that earthworms have a more enduring impact on boosting the fresh biomass of lettuce when organic fertilizer is used. As shown in Fig. 2, the effect of earthworms on increasing lettuce fresh biomass decreased from 21 % in the first season to 16 % in the third season with synthetic fertilizer addition, while it remained between 18 % and 21 % with organic fertilizer throughout the experiment. This difference may be partly explained by variations in the effect of earthworms on plant fertilizer N uptake, showing a stronger and longer-lasting effect with organic fertilizer.

Throughout the entire experiment, the increased N uptake from organic fertilizer by earthworms ranged between 10.08 and 11.24 mg/pot, indicating a long-lasting and stable effect of earthworms on promoting organic fertilizer mineralization and N release. This finding is supported by Table 1, which shows that the presence of earthworms led to higher inorganic-N content derived from organic fertilizer compared to treatments without earthworms throughout the experiment. Earthworms are known to enhance N mineralization from organic matter through both direct and indirect effects on the microbial community (Wu et al., 2018). Garnier et al. (2022) proposed several processes, including: (1) earthworms actively incorporate organic matter into the soil and fragment it into smaller pieces. This promotes organic N mineralization by increasing the surface area available for microbial colonization and further decomposition (Jiang et al., 2018), and (2) through ingestion, earthworms modify and stimulate microorganisms as they pass through the earthworm gut, resulting in differences in the microbial community in cast, which enables better exploitation of resources from organic matter (Chen et al., 2015). Regardless of the specific process, organic fertilizer N mineralization is primarily enhanced by earthworm within the drilosphere, the soil region influenced by earthworm activities such as digestion, casting, mucus secretion, and burrow formation (Brown et al., 2000). Clearly, the extension of drilosphere to the entire pots requires a certain amount of time, which explains why the positive effect of earthworms on organic fertilizer N release persists for a relatively long period (Lin et al., 2022).

In comparison, the effect of earthworms on increasing synthetic fertilizer uptake was very strong in the first season but sharply diminished in the second and third seasons. Urea is rapidly hydrolyzed into NH_4^+-N by soil urease (Fisher et al., 2016), making it an excellent short-

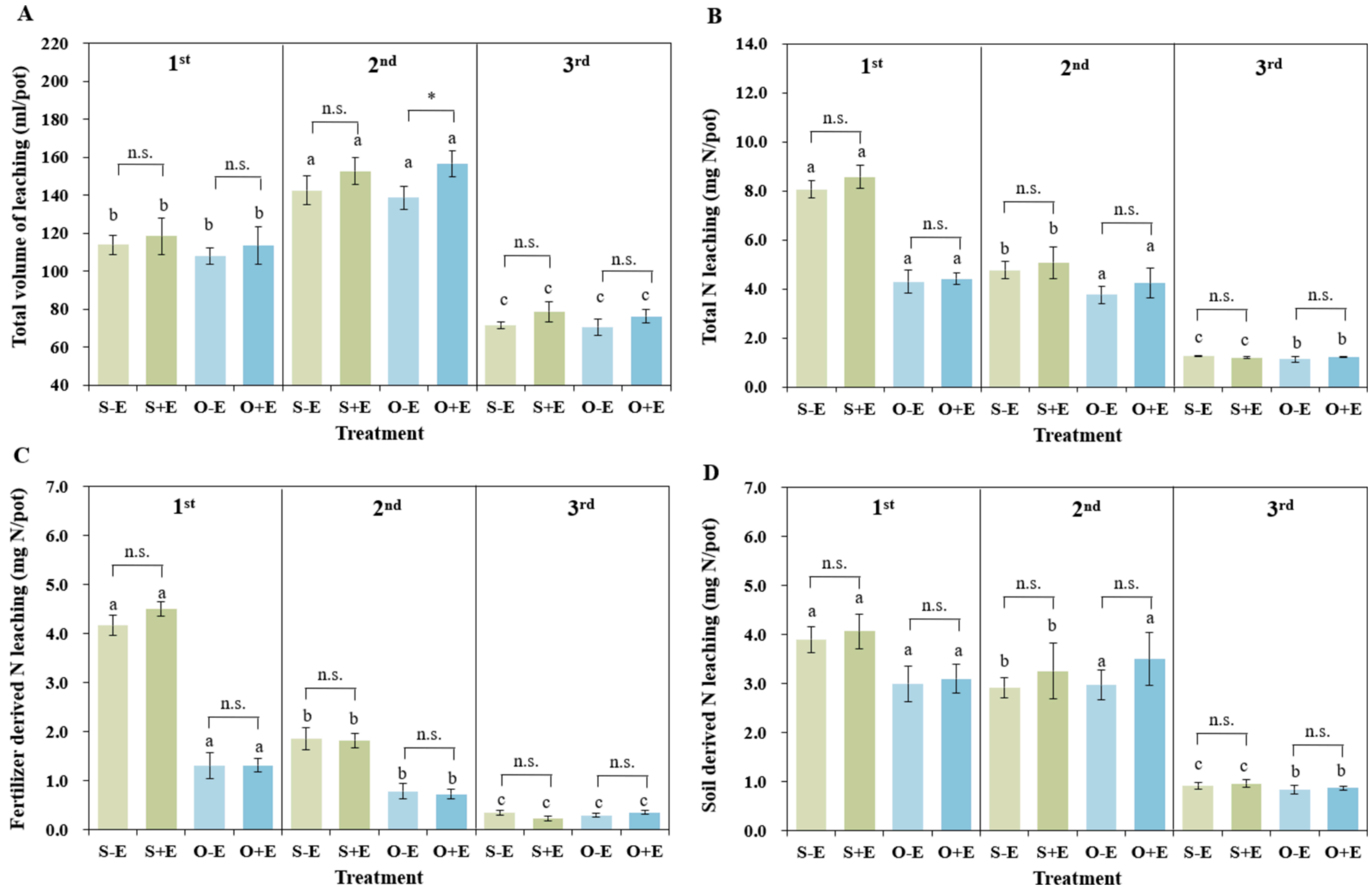

Fig. 5. Total Volumes of Leaching and Total N Losses by Leaching from Different Treatments (A) shows total volumes of leaching. (B), (C) and (D) shows total, fertilizer derived and soil derived N losses by leaching, respectively. 1st, 2nd, and 3rd denote the first, second, and third seasons of cultivation, respectively. Lowercase letters above the columns indicate significant differences ($P < 0.05$) between different seasons for the same treatment. Asterisks above black lines denote significant differences between earthworm presence and absence for the same fertilizer addition in each season (** significant at $P < 0.01$; * significant at $P < 0.05$; n.s. not significant). S + E and S-E indicate urea application with and without earthworms, respectively. O + E and O-E indicate compost application with and without earthworms, respectively.

term fertilizer for lettuce growth, with the plant deriving more than 70 % of its nitrogen nutrition from it during the first season. Earthworms increased the amount of synthetic fertilizer N uptake by plants during the first season because earthworm burrows assist plant roots in exploring a larger volume of soil (Springett and Gray, 1997), which means there was a greater surface area for nutrient (such as NH_4^+-N hydrolyzed from urea) acquisition (Chapman et al., 2012). This effect is particularly significant in the first season, as the soils were repacked and the role of earthworms in modifying rhizosphere soil structure is noticeable. In addition, earthworms may also promote plant growth by increased abundance and activity of beneficial micro-organisms in the soil (Hodson et al., 2023), reduced populations of pathogens (Euteneuer et al., 2019), production of plant growth promoting hormones (Binglei et al., 2023), and the modification of soil structure (Rossi, 2003), which thereby increasing plant N requirements and uptake. However, it should be note that the complete hydrolysis of urea applied to soils can take anywhere from 1 to 14 days (Fisher et al., 2016). This means that most of urea will have been transformed into other forms during the first season (40 days), either taken up by plants or lost to the environment. Consequently, only a relatively small percentage of synthetic fertilizer N remains available for plant uptake, leading to a sharp decrease in the effect of earthworms on fertilizer uptake in the following second and third seasons of cultivation. Nevertheless, the uptake of soil native N was significantly increased throughout the entire experiment by the presence of earthworms, due to the increased N mineralization from native soil organic matter (Postma-Blaauw et al., 2006).

4.2. Earthworms increased nitrogen loss from synthetic fertilizers but decreased nitrogen loss from organic fertilizers

Regardless of fertilizer type, earthworms increased total cumulative N_2O emissions by 14–25 % at the end of experiment, consistent with a previous meta-analysis that earthworm presence significantly increased soil N_2O emissions (Lubbers et al., 2013). Furthermore, the present study revealed that the increased N_2O emissions was primarily derived from soil native N rather than from fertilizer N. In addition to altering the activity and composition of soil microbes, earthworms enhance N_2O emissions by providing additional substrates for N_2O production through changes in the decomposition process of organic material (Wu et al., 2021). Obviously, the impact of earthworms on nitrogen mineralization from organic fertilizers may be obscured by the relatively small size of the fertilizer nitrogen pool (only 400 mg/pot) when compared to the larger native nitrogen pool in the soil (with a total nitrogen content of 17.31 g/kg). However, it is important to note that the presence of earthworms significantly increased N_2O emission derived from synthetic fertilizer N in the first season. This is likely due to the rapid hydrolysis of urea, which provided an abundant supply of ^{15}N substrates in the short-term, thereby facilitating the stimulation of microbes by earthworms and resulting in increased N_2O emissions.

NH_3 volatilization refers to the loss of nitrogen fertilizer to the atmosphere in the form of ammonia gas. After applying urea to the soil, a series of biochemical and chemical processes related to ammonia formation occurs rapidly (Li et al., 2022), leading to the primary NH_3

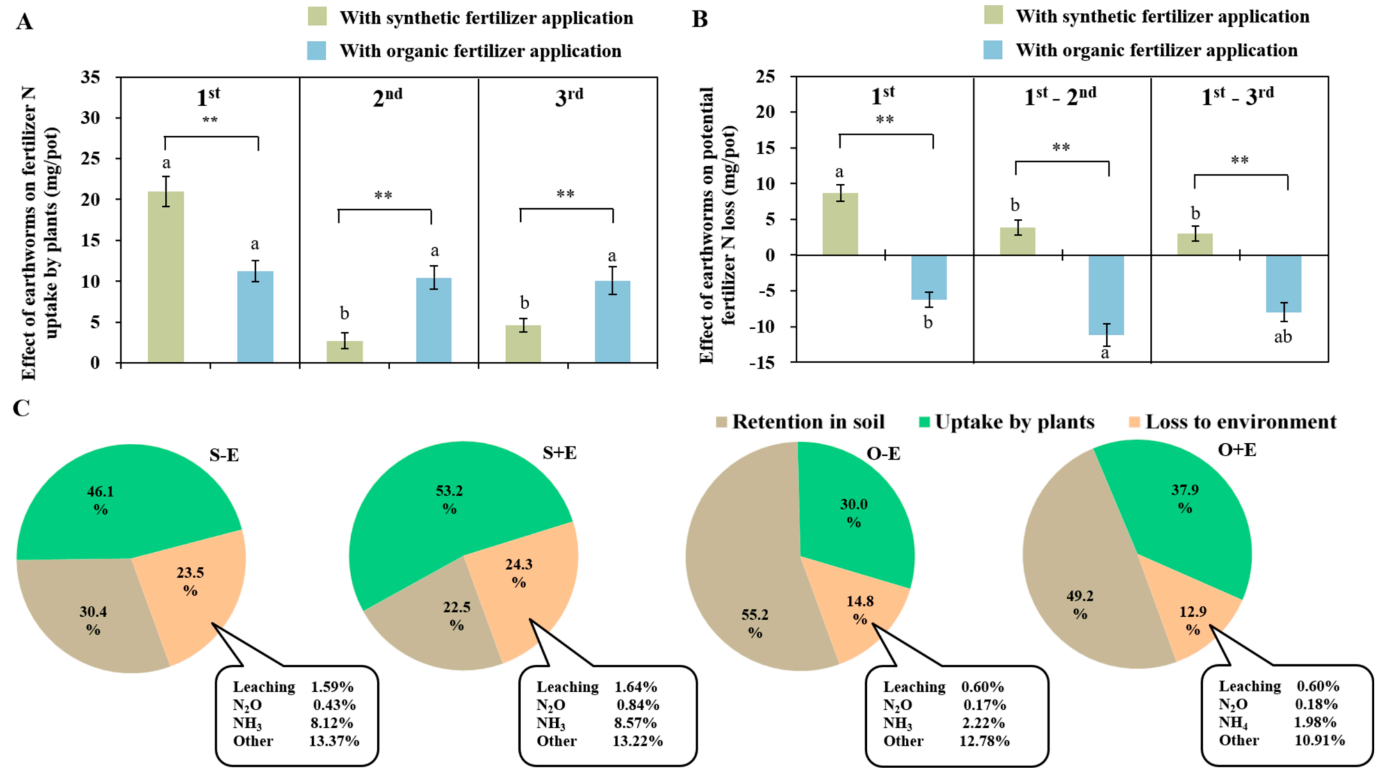

Fig. 6. Effect of Earthworms on Fertilizer N Uptake by Plants (A), Loss to the Environment (B), and the Fate of Fertilizer N at the End of the Experiment (C) In figure A, 1st, 2nd, and 3rd denote the first, second, and third seasons of cultivation, respectively. In figure B, potential N losses were estimated using a mass balance calculation method. Thus, the value of potential N losses encompassed N losses from both the current and previous seasons and shown as 1st, 1st-2nd and 1st-3rd. Lowercase letters above the columns indicate significant differences ($P < 0.05$) of the earthworms' effect between different seasons for the same fertilizer application. Asterisks above black lines denote significant differences ($P < 0.05$) of the earthworms' effect between different fertilizer application (** significant at $P < 0.01$; * significant at $P < 0.05$; n.s. not significant). S + E and S-E indicate urea application with and without earthworms, respectively. O + E and O-E indicate compost application with and without earthworms, respectively.

volatilization being detected in the first season with synthetic fertilizer. In contrast, NH_3 volatilization from organic fertilizer treatments was lower than that from synthetic fertilizer treatments, consistent with the findings of Wen et al. (2024), who further indicated that the most important factors influencing NH_3 volatilization are management practices, climate factors, and soil properties. In the current pot experiment, all the aforementioned factors were held consistent, resulting in no significant difference in cumulative ammonia (NH_3) volatilization between the treatments with and without earthworms.

By creating burrows and egesting casts, earthworms modify soil porosity, which impacts water infiltration and solute transport in the soil (Jouquet et al., 2012). However, most of determined factors shown in Fig. 5 did not exhibit a significant difference on leaching between treatments with and without earthworms. This may be attributed to the fact that the soil was repacked at the beginning of the experiment. Changes in soil porosity across the entire pot resulting from earthworm activity require more than a few weeks of cultivation to become evident. Additionally, the lower rainfall in the third season (339 mm) implies reduced potential for leachate generation, even though soil porosity has been improved by earthworm activity. Consequently, there was lower nitrogen recovery from fertilizer in the collected leachate.

After application, fertilizer N may be absorbed by plants, remain in the soil, or be lost to the environment in various forms, such as NH_3, N_2O, NO_x, N_2, leaching, runoff, etc. (Leach et al., 2012). Although half of the potential losses were accounted in the present study, it is difficult to determine all possible loss compartments of fertilizer N. Therefore, the potential total N losses in the present experiment were calculated as the difference between the total amount of applied N and the amount of recovered N from plants and soil. The presence of earthworms increased synthetic fertilizer N losses to the environment (primarily occurring in the first season) but decreased organic fertilizer N losses throughout the entire experiment, which partially contradicts Hypothesis 1. This differing effect may be attributed to the distinct transformation processes of fertilizer N in the soil. The hydrolysis of urea by urease is a critical initial step; subsequently, the hydrolyzed ammonium ion can dissolve in soil water and be lost through leaching, or be further converted into ammonia gas (NH_3) and other reactive nitrogen compounds (e.g., NO and N_2O) through nitrification and/or denitrification processes (Li et al., 2022). Earthworms have been reported to promote urease activity by 17.75 % to 121.91 % (Xu et al., 2021). The abundant release of ammonium ion in the short term, exceeding plant needs, can become a potential source of N loss. In contrast, the release of N from organic fertilizer relies on the decomposition process, which is much slower than the hydrolysis of urea. While earthworms accelerate organic matter decomposition by enhancing microbial activity and biomass (Huang et al., 2020), they also stimulate the utilization of released nutrients by microbes. This is evidenced by Table 1, which shows a significant increase in $MB^{15}N$ content with earthworm presence. Considering that the immobilization of fertilizer N in microbial biomass is a key process for retaining fertilizer N in the soil (Said-Pullicino et al., 2014), the increased $MB^{15}N$ content positively contributes to reducing organic fertilizer N loss. Additionally, the ability of earthworms to incorporate fresh organic matter into large soil macroaggregates may further reduce organic fertilizer N losses (Fahey et al., 2013).

4.3. *The fate of fertilizer nitrogen under earthworm activity*

Overall, throughout the experiment, earthworms had a greater effect on promoting the percentage of fertilizer N uptake by plants with organic fertilizer (an increase of 7.9 %) compared to synthetic fertilizer (an increase of 7.1 %). Given that nearly half of the organic fertilizer remains in the soil and considering the long-lasting effect of earthworms on organic fertilizer N release, this difference may become more pronounced with extended cultivation. This suggests that a longer-duration experiment is needed to fully elucidate the role of earthworms in agro-ecosystems, particularly with the addition of organic materials such as organic fertilizer and straw. Although earthworms increased the percentage of synthetic fertilizer N losses by 0.8 %, this is a minor proportion of the applied fertilizer and may be negligible in actual field conditions.

This study highlights the ecosystem services provided by earthworms based on the fate of the added fertilizer and suggests a greater benefit with organic fertilizer. However, it should be noted that changes in soil fertility depend on the balance between losses from the native soil N pool and the development of the new soil N pool following fertilization. In addition, with different earthworm species or different combinations of earthworm species, it is possible that some of the processes may be affected differently in a different soil (Andriuzzi et al., 2016). Additional studies, either considering the fate of soil native N or extending over a longer period with different earthworm species, are needed to investigate the effect of earthworms on soil N dynamics.

5. Conclusions

We concluded that the effect of earthworms (*Amynthas corticis*) on the fate of fertilizer N varies with the type of fertilizer applied. With synthetic fertilizer, earthworms increased the percentage of fertilizer N uptake by plants, especially during the first season of cultivation, while slightly increasing the percentage of fertilizer N loss to the environment. In contrast, with organic fertilizer, earthworms had a stronger and more sustained impact on promoting fertilizer N uptake by plants and reduced the percentage of fertilizer N loss to the environment. This study highlights the significant role of earthworms in fertilizer N dynamics with different types of fertilizer and suggests that organic fertilizer provides enhanced ecosystem services compared to synthetic fertilizer. Since nearly half of the organic fertilizer remains in the soil, a long-term experiment that incorporates changes in the soil native N pool is necessary to fully elucidate the role of earthworms in agro-ecosystem N cycling.

CRediT authorship contribution statement

Rong Hao: Formal analysis, Data curation, Conceptualization. **Yupeng Wu:** Writing – review & editing, Writing – original draft, Visualization, Supervision. **Hong Di:** Writing – review & editing. **Yunfeng Chen:** Writing – review & editing. **Weiguo Chen:** Writing – review & editing. **Ronggui Hu:** Supervision, Software, Resources. **Wenfeng Tan:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by National Natural Science Foundation of China (32171638) and National Key Research and Development Program of China (2021YFD1901202).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2024.117106.

Data availability

Data will be made available on request.

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