Effects of animal manure and nitrification inhibitor on N₂O emissions and soil carbon stocks of a maize cropping system in Northeast China

Dan Dong¹,²,³,⁴, Weichao Yang¹,⁴, Hao Sun¹, Shuang Kong¹ & Hui Xu¹

The incorporation of animal manure (AM) in soil plays an essential role in soil carbon sequestration but might induce higher soil nitrous oxide (N₂O) emissions. The use of nitrification inhibitors (NI) is an effective strategy to abate N₂O emission in agro-ecosystems. However, very few studies have evaluated the effectiveness of applying NI under the combined application of organic and inorganic fertilizers for increasing soil carbon sequestration and reducing N₂O emissions simultaneously in Northeast China. Here, a four-year field experiment was conducted with three treatments [inorganic fertilizer (NPK), inorganic fertilizer + manure (NPKM), and inorganic fertilizer with NI + manure (NPKI + M)], in a rainfed maize cropping system in Northeast China. Plots of different treatments were kept in the same locations for 4 years. Gas samples were collected using the static closed chamber technique, and nitrous oxide (N₂O) concentration in gas samples was quantified using a gas chromatograph. Soil organic carbon sequestration rate (SOCSR) was calculated based on the changes in SOC from April 2012 to October 2015. Averaged over the four years, AM incorporation significantly increased soil N₂O emissions by 25.8% \((p < 0.05)\), compared to NPK treatment. DMPP (3,4-dimethylpyrazole phosphate) significantly decreased N₂O emissions by 32.5% \((p < 0.05)\) relative to NPKM treatment. SOC content was significantly elevated by 24.1% in the NPKI + M treatment than the NPK treatment after four years of manure application \((p < 0.05)\). The annual topsoil SOCSR for the NPKM and NPKI + M treatments was 0.57 Mg ha⁻¹ yr⁻¹ and 1.02 Mg ha⁻¹ yr⁻¹, respectively, which were significantly higher than that of NPK treatment \((-0.61 \text{ Mg ha}^{-1} \text{ yr}^{-1}, p < 0.05).\) AM addition significantly increased the aboveground biomass and crop yields of maize in the fourth year. Overall, combined application of DMPP, inorganic fertilizer and AM is strongly recommended in this rainfed maize cropping system, which can increase maize yield and SOC sequestration rate, and mitigate N₂O emission.

In China, agricultural production generates \(2.4 \times 10^9\) tons of animal manure (AM) each year¹. The application of AM to soil can help to slow climate change by increasing soil carbon sequestration², improve soil fertility, and tackle environmental problems associated with nitrogen-rich waste management³. Nevertheless, AM amendment might cause substantial nitrous oxide \((\text{N}_2\text{O})\) emissions from soils. Intensively fertilized upland soil is one of the anthropogenic sources of N₂O, and the GWP (Global Warming Potential) of N₂O is 298 times than that of CO₂ over a century time horizon⁴. Application of AM will alter soil aerobic conditions, pH, and porosity, and then affect N₂O emission⁵. It is typically believed that, in comparison to inorganic fertilizers, AM provides more labile organic carbon sources for soil microbes, thereby stimulating N₂O emission from nitrification and denitrification. A global meta-analysis found that the increases in N₂O emissions caused by manure application

¹Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China. ²Jiangsu Collaborative Innovation Center of Regional Modern Agriculture and Environmental Protection/Jiangsu Key Laboratory for Eco-Agriculture Biotechnology Around Hongze Lake, Huaiyin Normal University, Huai’an 223300, China. ³University of Chinese Academy of Sciences, Beijing 100049, China. ⁴These authors contributed equally: Dan Dong and Weichao Yang. ⁵email: xuhui@iae.ac.cn
might offset the benefit of increasing soil organic carbon (SOC) stocks. In order to mitigate the emission of \( \text{N}_2\text{O} \), sustainable agricultural practices must be explored and carried out.

Nitrification inhibitors (NI) have been suggested as a potential option to mitigate agricultural soil \( \text{N}_2\text{O} \) emissions by the Intergovernmental Panel on Climate Change. As a recommended NI, 3,4-dimethylpyrazole phosphate (DMPP) has been proved effective at reducing \( \text{N}_2\text{O} \) emissions from croplands, although the reported abatement of \( \text{N}_2\text{O} \) emissions ranged from 22 to 77% in maize cropping systems. Furthermore, different AM types and managements can make a big difference in the size of subsequent \( \text{N}_2\text{O} \) emissions. In addition, \( \text{N}_2\text{O} \) emission is also affected by soil characteristics, climatic conditions, and crop management. Although several studies have measured the effects of AM-based soil amendments on \( \text{N}_2\text{O} \) emissions from maize cropping systems in Northeast China—31% of the national maize is grown in the region—most of these studies quantified \( \text{N}_2\text{O} \) emissions less than one year, which can’t fully capture the inter-annual characteristics of \( \text{N}_2\text{O} \) emissions. Due to lack of long-term measurement under AM applications, there is still great uncertainty about the quantification and mitigation of \( \text{N}_2\text{O} \) emissions in the maize cropping system.

To address these gaps, this study presented a long-term observation of \( \text{N}_2\text{O} \) emission and soil carbon sequestration in a maize cropping system in Northeast China. The main objectives of this study were: (1) to evaluate the combined application of inorganic fertilizer and AM on \( \text{N}_2\text{O} \) emissions and soil organic carbon sequestration; and (2) to test if DMPP can effectively reduce \( \text{N}_2\text{O} \) emission and increase soil organic carbon sequestration under the combined application of inorganic fertilizer and AM.

**Materials and methods**

**Study area and soil properties.** A field experiment was established in May 2012 at Shenyang Agro-Ecological Station (41°31’N, 123°22’E) of the Institute of Applied Ecology, Chinese Academy of Sciences, Northeast China. This region has a warm-temperate continental monsoon climate. The mean annual air temperature and annual precipitation are 7.5 °C and 680 mm, respectively. The soil is classified as Luvisol (FAO classification).

The soil properties of the topsoil layer (0–20 cm) at the start of the experiment are as follows: SOC = 9.0 g kg⁻¹, available \( \text{NH}_4^+ \text{N} = 1.18 \text{ mg kg}^{-1} \), available \( \text{NO}_3^-\text{N} = 9.04 \text{ mg kg}^{-1} \), Olsen-P = 38.50 mg kg⁻¹, available K = 97.90 mg kg⁻¹, bulk density = 1.25 g cm⁻³, and pH = 5.8. The determination method of soil was shown in “Soil analysis” section.

**Field experiment.** Three treatments were established in this experiment: (1) mineral fertilizers (NPK); (2) pig manure incorporation at a local conventional AM application rate of 15 Mg ha⁻¹ yr⁻¹ (NPKM, 126 kg N ha⁻¹ on dry weight); and (3) NPKM plus DMPP (3,4-Dimethylpyrazole phosphate) incorporation at a rate of 0.5% of applied urea (2.39 kg ha⁻¹, 220 kg N/the N content of urea (0.46 × 0.5%) (NPKM + M). The treatments were applied following a randomized design across three replicate field plots (4 m × 5 m). Plots of different treatments remained unchanged in the same locations for 4 years. Each year, the composted pig manure (213 g C kg⁻¹ and 22 g N kg⁻¹ based on dry weight on average, characteristics of pig manure was listed in Table S1) was broadcasted evenly onto the plots a few days before maize planting, and ploughed to a depth of 20 cm by machine (TG4, Huaxing, China). For the respective treatments, urea (220 kg N ha⁻¹ yr⁻¹), calcium superphosphate (110 kg P₂O₅ ha⁻¹ yr⁻¹), and potassium chloride (110 kg K₂O ha⁻¹ yr⁻¹) were applied on the same day as maize (Zea mays L.) was planted. The urea and inhibitor were fully mixed before application.

Maize (cultivar was Fuyou #9) was planted on 3rd May 2012, 3rd May 2013, 6th May 2014, and 10th May 2015, at a spacing of 37 cm and 60 cm between rows. No irrigation was applied throughout the experimental period. Maize was harvested on 13th September 2012, 29th September 2013, 29th September 2014, and 29th September 2015, respectively. At harvest, maize yield and aboveground biomass yield were measured by harvesting all plants (20 m²) in each plot. The straw and grain were removed after each harvest and the soil with about 5 cm maize stem was ploughed to a depth of approximately 20 cm in April each year.

Each cropping cycle, therefore, consisted of periods of maize (from May to September) and fallow (from October to April) of the following year.

The precipitation and air temperature data were acquired from the meteorological station of the Shenyang Agro-Ecological Station. The precipitation during the 2012/2013, 2013/2014, 2014/2015, and 2015/2016 periods were 911.9 mm, 621.7 mm, 485.7 mm, and 585.3 mm, respectively (Fig. 1). 72.3%, 75.5%, 66.5%, and 73.0% of these annual precipitations occurred during maize-growing period, respectively. The mean annual air temperatures in these years were 7.7 °C (− 21.2 to 27.5 °C), 8.1 °C (− 22.7 to 28.3 °C), 9.5 °C (− 21.7 to 28.2 °C) and 9.3 °C (− 17.1 to 27.0 °C), respectively. The soil temperature at a depth of 5 cm varied between − 14 and 35 °C during the four-year period (Fig. 2b). The change trend of soil surface temperature was the same as that of soil temperature at 5 cm depth (Fig. 2a). The mean soil WFPS (0–15 cm) varied between 15 and 73% (Fig. 2c).

**Gas sampling and analysis.** The gas was sampled between 3rd May 2012 and 14th April 2016 using a static closed chamber system as described by Dong et al. Briefly, a stainless-steel chamber base (56 cm length × 28 cm width) was inserted into the soil of each plot to a depth of approximately 10 cm, with its long edge perpendicular to the rows of maize. The top chamber (56 cm length × 28 cm width × 20 cm height) was also made of stainless steel. Gas samples were obtained using a syringe 0, 20, and 40 min after the chambers had been closed between 9:00 am and 11:00 am on each sampling day. Gas samples were collected every 2–6 days and every 7–15 days during the growing seasons and non-growing seasons, respectively. The first gas sampling time was on day 1, day 3, day 1, and day 3 after maize planting each year. The \( \text{N}_2\text{O} \) concentrations in gas samples were quantified using a gas chromatograph (Agilent 7890A, Shanghai, China) with an electron capture detector.
Soil analysis. The soil temperature and volumetric water content (SVWC) were measured at depth of 0–15 cm using a bent stem thermometer and a time-domain reflectometry (Zhongtian Devices Co. Ltd, China), respectively. SVWC was converted to soil water-filled pore space (WFPS) using the following equation:

\[
WFPS = \frac{SVWC}{1 - \frac{BD}{\text{particle density}}}
\]

where BD is soil bulk density (g cm\(^{-3}\)). Particle density was assumed to be 2.65 g cm\(^{-3}\).
Soil samples from the 0–20 cm layer were collected in each plot in April 2012 (before sowing) and October 2015 (maize harvest) using a 5 cm diameter stainless steel soil sampler. The five soil samples collected from different locations in each plot were mixed thoroughly. Visible roots were removed by hand and the samples were air-dried and sieved using a 0.15 mm sieve. SOC was then quantified using a elemental analyzer (Vario EL III, Elementar, Germany). Soil available NH₄⁺–N and NO₃⁻–N were extracted with 2 M KCl and measured colorimetrically using a continuous flow injection analyzer (Futura, Alliance, France). Soil Olsen-P was extracted with NaHCO₃ and colorimetrically measured using a spectrophotometer (Lambda 2, PerkinElmer, USA). Soil available K was extracted by 1 M CH₃COONH₄ and analyzed with a flame photometer (FP640, Jingmi, China). Soil pH was determined with deionized water (1:2.5) and analyzed using a pH meter (PHS-3C, LeiCi, China) with a glass electrode.

**DNA extraction and real-time quantitative PCR.** The soil samples for measuring the abundance of nitrification and denitrification functional genes were collected on May 20, 2015. Soil DNA was extracted with the soil DNA extracted kits (EZNA soil DNA Kit; Omega Bio-Tek Inc., U.S.A.). The copy numbers of nitrification and denitrification functional genes were determined by q-PCR with the Roche LightCyler® 96 (Roche, Switzerland). Additional details about the primers and amplification procedure can be found in Dong et al.16.

**Data analysis.** The N₂O flux (μg N₂O–N m⁻² h⁻¹) is calculated based on the increase of N₂O concentration per unit chamber area for a specific time interval18 as follows:

\[
F = 273/(273 + T) \times M/22.4 \times H \times \frac{dc}{dt} \times 1000
\]

(2)

where F (μg N₂O–N m⁻² h⁻¹) is the N₂O flux, T (°C) is the air temperature in the chamber, M (g N₂O–N mol⁻¹) is the molecular weight of N₂O–N, 22.4 (L mol⁻¹) is the molecular volume of the gas at 101.325 kPa and 273 K, H (m) is the chamber height, dc/dt (ppb h⁻¹) is the rate of change in the N₂O concentration in the chamber.

Cumulative N₂O emissions were calculated as follows:

\[
\text{Cumulative emission} = \sum_{i=1}^{n} \left( \frac{F_i + F_{i+1}}{2} \times (t_{i+1} - t_i) \right) \times 24
\]

(3)

where F is the N₂O emission flux (μg N₂O–N m⁻² h⁻¹), i is the ith measurement, (tᵢ₊₁ − tᵢ) is the number of days between two adjacent measurements, and n is the total number of the measurements. Annual N₂O emissions were calculated between the fertilization dates of each successive year.

The SOC stock (Mg ha⁻¹) in the topsoil was calculated as:

\[
C_{\text{stock}} = \text{SOC} \times BD \times D \times 10,
\]

(4)

where BD is soil bulk density (g cm⁻³), D is the depth of the topsoil (0.2 m).

The topsoil SOC sequestration rate (SOCR) (Mg ha⁻¹ yr⁻¹) was estimated using the following equation:

\[
\text{SOCR} = \frac{(C_{\text{stock}2015} - C_{\text{stock}2012})}{t},
\]

(5)

where C_{stock2015} and C_{stock2012} are the SOC stocks in 2015 and 2012, respectively, and t is the duration of the experiment (years).

Statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, USA). The differences in cumulative N₂O emissions and maize yields within a year, and other factors among treatments were assessed using one-way Analysis of Variance (ANOVA) with least significant difference post-hoc tests and a 95% confidence limit. The effects of different treatments, years, and their interactions on N₂O emission, maize yield and aboveground biomass were examined using one-way repeated measures ANOVA. Pearson correlation analysis was used to analyze the relationships between cumulative N₂O emissions and precipitation (N = 12 (three data each year, four years)), as well as N₂O flux and soil available nitrogen content.

**Statements of research involving plants.** It is stated that the current research on the plants comply with the relevant institutional, national, and international guidelines and legislation. It is also stated that the appropriate permissions have been taken wherever necessary, for collection of plant or seed specimens. It is also stated that the authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

**Results**

**Soil mineral N.** Soil NH₄⁺–N and NO₃⁻–N concentrations were shown in Fig. S1. The contents of soil NH₄⁺–N and NO₃⁻–N increased significantly after fertilization, and gradually decreased after reaching the maximum value. No significant difference in soil NH₄⁺–N concentrations was found between NPKM and NPKI + M treatments except August 13th 2012. Soil NO₃⁻–N contents of NPKI + M treatment on June 12th and July 4th 2014 were significantly higher than that of the NPKM treatment.

**Maize grain yield and aboveground biomass.** Across the four-year observation period, although the yearly average of maize yield of AM amendment treatment (NPKM and NPKI + M) had an increasing trend relative to NPK treatment, the repeated measurement analysis of variance showed that the difference between these treatments was not significant (p > 0.05, Table 1). However, the grain yields were significantly increased
in AM amendment treatments (NPKM and NPKI+M) in the fourth year (2015) (Table 1), compared to NPK treatment. During the four-year period, aboveground biomasses of the NPKM and NPKI+M treatments were significantly higher relative to the NPK treatment, by 12.0% and 10.3%, respectively ($p < 0.05$, Table 1). Through repeated-measures ANOVA, the significant interaction was not found between observation years and treatments effect on aboveground biomass.

N$_2$O flux and related nitrification and denitrification gene abundance. Seasonal variations in soil N$_2$O flux are shown in Fig. 3. The highest N$_2$O fluxes typically occurred after fertilizer application and occasionally coincided with freeze–thaw events in 2012/2013 (Fig. 3). The highest N$_2$O flux ($235.6$ μg m$^{-2}$ h$^{-1}$) was observed from the NPKM treatment plot on May 27th 2014, while it was significantly mitigated by the DMPP amendment in NPKI+M treatment ($51.3$ μg m$^{-2}$ h$^{-1}$).

Relative to the NPK treatment, the cumulative N$_2$O emissions from the NPKM plot was significantly increased by 25.8% on average ($p < 0.05$, Table 2). However, in three of four years, N$_2$O emissions were not statistically different between the NPK and NPKM treatments. During 2014/2015, NPKM increased N$_2$O emissions by 63.0% relative to the NPK treatment ($p < 0.05$). Compared to the NPKM treatment, the addition of DMPP (NPKI+M) significantly decreased annual N$_2$O emissions by 51.9%, 54.4%, and 22.5% in 2013/2014, 2014/2015 and 2015/2016, respectively. Across the four-year observation period, the N$_2$O emissions decreased by 32.5% in NPKI+M treatment, compared with the NPKM treatment (Table 2).

Significant linear negative relationships between precipitation and N$_2$O emission in growing season ($N = 12$, $p < 0.05$) and significant positive relationships between precipitation and N$_2$O emission in non-growing season were found ($N = 12$, $p < 0.01$). Correlation analysis showed that N$_2$O emission fluxes had a very significant positive correlation with the contents of NH$_4$$^+$–N and NO$_3$$^−$–N in soil.

| Treatments | Grain yields | Aboveground biomass |
|------------|--------------|---------------------|
|            | 2012         | 2013         | 2014         | 2015         | Mean | 2012         | 2013         | 2014         | 2015         | Mean |
| NPK        | 11.62 ± 0.54 b | 11.74 ± 0.88 a | 11.60 ± 0.92 b | 11.38 ± 0.37 b | 11.58 ± 0.44 a | 23.22 ± 1.11 a | 22.09 ± 1.63 a | 20.41 ± 2.07 b | 22.44 ± 0.88 b | 22.04 ± 0.84 b |
| NPKM       | 12.27 ± 0.25 ab | 13.13 ± 0.75 a | 12.60 ± 0.36 a | 12.96 ± 0.47 a | 12.70 ± 1.08 a | 24.70 ± 1.32 a | 26.11 ± 1.09 a | 24.20 ± 0.63 a | 24.68 ± 0.48 a |
| NPKI+M     | 12.55 ± 0.53 a | 12.36 ± 1.53 a | 13.32 ± 0.82 ab | 12.29 ± 0.44 a | 12.63 ± 0.76 a | 24.18 ± 2.46 a | 23.70 ± 1.97 a | 25.11 ± 0.86 a | 24.23 ± 0.56 a | 24.31 ± 1.25 a |

Table 1. Maize grain yields and aboveground biomass from 2012 to 2015 (Mg ha$^{-1}$). Different lowercase letters indicate significant differences ($p < 0.05$). “with” the same letters were not significantly different ($p > 0.05$).
The results of nitrification and denitrification functional gene abundance were shown in Table 3. Compared with NPK, NPKM significantly increased the AOB \textit{amoA} and \textit{nosZ} gene abundance by 88% and 172%, respectively. There was no significant difference in AOB \textit{amoA} and \textit{nosZ} gene abundance between NPK and NPKI + M treatments.

**Soil organic carbon sequestration rate.** The SOC content was 9.0 g kg\(^{-1}\) at the beginning of the experiment in 2012. Relative to the NPK treatment, SOC content was significantly elevated (by 24.1%) in the NPKI + M treatment after four years of manure application (\(p < 0.05\)). The annual topsoil SOCSR for the NPKM and NPKI + M treatments was 0.57 Mg ha\(^{-1}\) yr\(^{-1}\) and 1.02 Mg ha\(^{-1}\) yr\(^{-1}\), respectively (Table 4). Compared to the NPK treatment, the NPKM and NPKI + M treatments significantly increased SOCSR, respectively (\(p < 0.05\), Table 4).

**Discussion**

\textbf{N}_2\text{O} emissions. Large inter-annual variations in N\(_2\)O emissions were observed during the study period. Xia et al. and Cayuela et al. reported that N\(_2\)O emission is affected by soil characteristics, climatic conditions, and crop management measures\(^{19,20}\). In this study, according to the relationships between precipitation amount and N\(_2\)O emissions, the precipitation amount might be one of the most important controlling factors on N\(_2\)O emissions, especially in the AM addition treatment. Meanwhile, the precipitation distribution might also be an important factor for N\(_2\)O flux. There was a positive correlation between N\(_2\)O flux and soil available N (NH\(_4\)^+–N and NO\(_3\)^−–N), indicating that the coupling of water and nitrogen was one of the reasons for the higher N\(_2\)O emissions. Generally speaking, precipitation before and after the fertilization period (plenty available N as shown in Fig. S1) is prone to cause higher N\(_2\)O emissions, such as in 2014/2015. While in the later growing season (less available N as shown in Fig. S1), even if large precipitation happened, it will not cause higher N\(_2\)O emissions, such as in August of each year. This may be because the continuous consumption of N in the soil (such as absorption by maize, volatilization, and runoff, etc.) resulted in a decrease in available N in the soil, which ultimately reduced the release of N\(_2\)O. Therefore, the results of our study showed that the distribution and amount of precipitation had a significant effect on N\(_2\)O emissions in a rainfed cropping system, which is consistent with the results reported in previous studies\(^{21}\).

In average over 4 years, the addition of AM (NPKM) significantly increased soil N\(_2\)O emissions relative to the control treatment (NPK), which is consistent with previous studies\(^{14,22}\). Specifically, N\(_2\)O emissions were 63.0% higher with the addition of AM (NPKM) in 2014/2015 (\(p < 0.05\)). The higher N\(_2\)O emission recorded for the NPKM treatment might be explained with two key mechanisms: Firstly, the total N input is higher in the

| Treatment | 2012–2013 | 2013–2014 | 2014–2015 | 2015–2016 | Mean |
|-----------|-----------|-----------|-----------|-----------|------|
| NPK       | 1.05 ± 0.17 a | 0.40 ± 0.14 a | 1.04 ± 0.27 b | 0.87 ± 0.12 a | 0.84 ± 0.07 b |
| NPKM      | 1.26 ± 0.20 a | 0.41 ± 0.08 a | 1.70 ± 0.40 a | 0.86 ± 0.01 a | 1.06 ± 0.05 a |
| NPKI + M  | 1.22 ± 0.07 a | 0.20 ± 0.06 b | 0.78 ± 0.19 b | 0.66 ± 0.01 b | 0.71 ± 0.06 c |

| Treatment | AOA \textit{amoA} | AOB \textit{amoA} | \textit{nirS} | \textit{nirK} | \textit{nosZ} |
|-----------|----------------|----------------|---------|---------|---------|
| NPK       | 9.73E + 06 a | 4.87E + 06 b | 3.17E + 06 a | 5.17E + 05 b | 4.55E + 06 b |
| NPKM      | 1.46E + 07 a | 9.16E + 06 a | 3.88E + 06 a | 1.20E + 06 ab | 1.24E + 07 a |
| NPKI + M  | 1.17E + 07 a | 5.88E + 06 ab | 3.46E + 06 a | 1.27E + 06 a | 6.37E + 06 b |

| Treatment | TN (g kg\(^{-1}\)) | SOC (g kg\(^{-1}\)) | C\(_{\text{stock}}\) (Mg ha\(^{-1}\)) | SOCSR\(^a\) (Mg C ha\(^{-1}\) yr\(^{-1}\)) |
|-----------|-----------------|-----------------|----------------|-----------------|
| NPK       | 0.93 ± 0.04 b | 8.40 ± 0.57 b | 20.99 ± 1.42 b | −0.61 ± 0.38 b |
| NPKM      | 1.06 ± 0.09 a | 9.64 ± 0.77 ab | 24.10 ± 1.92 ab | 0.57 ± 0.45 a |
| NPKI + M  | 1.01 ± 0.06 ab | 10.42 ± 0.69 a | 26.05 ± 1.72 a | 1.02 ± 0.44 a |

| Treatment | 2012–2013 | 2013–2014 | 2014–2015 | 2015–2016 | Mean |
|-----------|-----------|-----------|-----------|-----------|------|
| NPK       | 1.05 ± 0.17 a | 0.40 ± 0.14 a | 1.04 ± 0.27 b | 0.87 ± 0.12 a | 0.84 ± 0.07 b |
| NPKM      | 1.26 ± 0.20 a | 0.41 ± 0.08 a | 1.70 ± 0.40 a | 0.86 ± 0.01 a | 1.06 ± 0.05 a |
| NPKI + M  | 1.22 ± 0.07 a | 0.20 ± 0.06 b | 0.78 ± 0.19 b | 0.66 ± 0.01 b | 0.71 ± 0.06 c |

The SOCSR was estimated from April 2012 to October 2015. Values followed by different lowercase letters at the same column indicated significant difference (\(P < 0.05\)) among the treatments.

Table 2. Annual cumulative fluxes of N\(_2\)O (kg N ha\(^{-1}\)) under different treatments through the experimental period (2012–2015). Mean ± standard deviation (n = 3). Different lowercase letters in one column indicate significant difference among treatments (\(p < 0.05\)).

Table 3. Ammonia oxidizers and denitrifier functional gene abundance (copies g\(^{-1}\) of dry soil). Values followed by different lowercase letters at the same column indicated significant difference (\(P < 0.05\)) among the treatments.

Table 4. TN, SOC, C\(_{\text{stock}}\) and SOCSR at 0–20 cm soil depth after four year’s different fertilization treatments. \(^a\)The SOCSR was estimated from April 2012 to October 2015. Values followed by different lowercase letters at the same column indicated significant difference (\(P < 0.05\)) among the treatments.
NPKM treatment (mean = 346 kg N ha⁻¹) than in the NPK treatment (mean = 220 kg N ha⁻¹). Previous studies have reported a positive correlation between nitrogen application rates and N₂O emissions, although cumulative N₂O emission may have an upper threshold under increasing organic nitrogen inputs. Secondly, the long-term organic manure application can increase the total organic C and soil availability of DOC, which could stimulate microbial activity and N₂O production in soil.

In three of the four observation years, cumulative N₂O emissions did not differ between the NPK and NPKM treatments despite the much greater N application in the NPKM plot, and this phenomenon is consistent with previous studies. Organic fertilizer provides organic C substrate for microbial growth, so it promotes microbial N assimilation. This effect usually leads to a strong competition for NH₄⁻ between heterotrophic microorganisms and autotrophic nitrifiers, mitigating the yield of N₂O. However, the input of organic C and N may promote the growth of active microorganisms and consume O₂ in soil pores, resulting in the formation of micro-an aerobic environments, stimulate denitrification and produce N₂O. In this study, the NPKM treatment increased the occurrence of the nosZ gene by 172% (supplementary materials, Table 3), relative to the NPK treatment, indicating a higher proportion of N₂O had been reduced to N₂ in NPKM treatment. Meanwhile, the higher AOB amoA gene was also found in NPKM treatment, which might induce much N₂O formation. Therefore, considering the combined effects of the above nitrification and denitrification, there was no significant difference in N₂O emissions between NPK and NPKM in 2015 in our study. Overall, our results suggest that, in the rainfed maize cropping system, the combined application of inorganic fertilizer and AM might promote the emission of N₂O in comparison to inorganic fertilizer applied alone.

The application of NPKM (NPK + M) significantly decreased cumulative N₂O emissions relative to the NPKM treatment, which is consistent with previous studies. The observed percentage in N₂O emissions reduction ranged between 22.5% and 54.4%, which is comparable to other studies applying DMPP including a reduction of 24% reported by Huérfano et al. and 53% reported by Weiske et al. Based on a review of the literature on N₂O application, Akiyama et al. reported that the application of N reduces N₂O emissions by an average of 38%. Furthermore, Qiao et al. reported that N application could increase N₂O emissions by 20%. Our results showed N₂O losses were lower in the NPKM treatment than in the NPK treatment. In addition, SOC and maize biomass, a large part of the applied N was stored in the soil according to TN data (Table 4). Further studies should be conducted to investigate the long-term application of AM on N loss in a maize-soil system.

The addition of DMPP (NPKI + M) significantly decreased cumulative N₂O emissions relative to the NPKM treatment, which is consistent with previous studies. The observed percentage in N₂O emissions reduction ranged between 22.5% and 54.4%, which is comparable to other studies applying DMPP including a reduction of 24% reported by Huérfano et al. and 53% reported by Weiske et al. Based on a review of the literature on N₂O application, Akiyama et al. reported that the application of N reduces N₂O emissions by an average of 38%. Furthermore, Qiao et al. reported that N application could increase N₂O emissions by 20%. Our results showed N₂O losses were lower in the NPKM treatment than in the NPK treatment. In addition, SOC and maize biomass, a large part of the applied N was stored in the soil according to TN data (Table 4). Further studies should be conducted to investigate the long-term application of AM on N loss in a maize-soil system.

Maize yield and SOCSR. Addition of AM significantly increased (10.7% and 8.0% for NPKM and NPKI + M, respectively) the maize yields in the fourth year, which is comparable to the study of Li et al. conducted in Northeast China. On one hand, there was more N provided in AM amendment treatment in comparison to NPK treatment. On the other hand, the organic form of N was released later in the growing season of maize (especially in 2014 and 2015, Fig. S1), which provided a better match between N supply and maize requirement in comparison to NPK treatment. In comparison, maize yields were not significantly affected by DMPP application, as has also been reported.

The results showed that long-term application of inorganic fertilizers induced the loss of SOC, since C inputs obtained only from maize residue were smaller than C loss in inorganic fertilizer treatment. It has also been reported in other studies in Northeast China, in which a declined SOC was found in inorganic fertilizer treatment. Therefore, in our opinion, for the sustainable development of agriculture in Northeast China, it is necessary to apply AM with inorganic fertilizers. The annual SOCSR in this study was similar to a multi-site study of manure application in a mono-cropping system reported by Zhang et al. and a soybean and maize rotation system in Northeast China by Ding et al. The results suggest that the sequestration of SOC might be mainly associated with the direct C supply from AM and the indirect C supply through higher maize yields. Application of organic manure is an effective agricultural practice for enhancing SOC storage in the maize cropping system. It is necessary to further study the processes and mechanisms of SOC sequestration induced by DMPP application.

Based on the results of maize grain yield and aboveground biomass, NPKM would be used to achieve higher maize yield and aboveground biomass, but it would increase N₂O emission of maize production. Compared with NPK, NPKM did not significantly increase the content of SOC, while SOC were significantly increased by combined inorganic and organic fertilizer application with DMPP. The results of this study suggest that increasing SOC and maize yield, as well as N₂O mitigation can be simultaneously achieved by the combined application of inorganic and organic fertilizer with DMPP. It is necessary to measure the changes of SOC and N₂O emissions at the same time when formulating the optimal management measures for sustainable maize production.
Conclusions

Long term application of inorganic fertilizers led to the loss of SOC. Generally speaking, applying animal manure is considered to be an effective way to improve soil SOC. However, there is a risk of enhanced N₂O emission with manure application. Through a consecutive four-year field experiment on Luvisol soil in Northeast China, our results showed that the combined application of NI, such as DMPP, inorganic fertilizer and animal manure into soil should be recommended in Northeast China, as it could not only mitigate N₂O emissions but also increase maize yield and SOC sequestration rate.

Received: 25 October 2021; Accepted: 31 August 2022
Published online: 08 September 2022

References

1. Wang, Z. et al. Livestock manure resources and their replace potential fertilizer in China. Chin. Agric. Sci. Bull. 35(26), 121–128 (2019).
2. Li, C., Froliking, S. & Butterbach-Bahl, K. Carbon sequestration in arable soils is likely to increase nitrous oxide emissions, offsetting reductions in climate radiative forcing. Clim. Change 2107(23), 321–338 (2005).
3. Aguilera, E. et al. The potential of organic fertilizers and water management to reduce N₂O emissions in Mediterranean climate cropping systems. A review. Agric. Ecosyst. Environ. 164, 32–52 (2013).
4. IPCC. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. 2007, Cambridge University Press.
5. Cui, P. Y. et al. Long-term organic and inorganic fertilization alters temperature sensitivity of potential N₂O emissions and associated microbes. Soil Biol. Biochem. 93, 131–141 (2016).
6. Velthof, G. L., Klute, A, Methods of Soil Analysis, Part 1, Physical and Mineralogical Methods. Agromomy Monographs Society of Agronomy, 1982).
7. Zhou, M. et al. Stimulation of N₂O emission by manure application to agricultural soils may largely offset carbon benefits: A global meta-analysis. Glob. Change Biol. 23(10), 4068–4083 (2017).
8. IPCC. Climate Change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. O. Edenhofer, et al., Editors, 2014, Cambridge University Press. p. 1281–1304.
9. Weiske, A. et al. Influence of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in comparison to dicynandiamide (DCD) on nitrous oxide emissions, carbon dioxide fluxes and methane oxidation during 3 years of repeated application in field experiments. Biol. Fertil. Soils 34(2), 109–117 (2001).
10. Huerfano, X. et al. DMPSA and DMPP equally reduce N₂O emissions from a maize-ryegrass forage rotation under Atlantic climate conditions. Atmos. Environ. 187, 255–265 (2018).
11. Iu, X. et al. Processes and factors controlling N₂O production in an intensively managed low carbon calcareous soil under sub-humid monsoon conditions. Environ. Pollut. 159(4), 1007–1016 (2011).
12. Halvorson, A. D., Del Grosso, S. J. & Stewart, C. E. Manure and inorganic nitrogen affect trace gas emissions under semi-arid irrigated corn. J. Environ. Qual. 45(3), 906–914 (2016).
13. Tan, Y. et al. Effects of optimized N fertilization on greenhouse gas emission and crop production in the North China Plain. Field Crop. Res. 205, 135–146 (2017).
14. Li, L. et al. Nitrous oxide emissions from Mollsols as affected by long-term organic amendments and chemical fertilizers. Sci. Total Environ. 452, 302–308 (2013).
15. Stehfest, E. & Bouwman, L. N₂O and NO emission from agricultural fields and soils under natural vegetation: Summarizing available measurement data and modeling of global annual emissions. Nute. Cycl. Agroecosyst. 74(3), 207–228 (2006).
16. Dong, D. et al. Effects of urease and nitrification inhibitors on nitrous oxide emissions and nitrifying/denitrifying microbial communities in a rainfed maize soil: A 6-year field observation. Soil Till. Res. 180, 82–90 (2018).
17. Miller, R. H. and D. R. Keeny. Methods of soils analysis, part 2. In Chemical and Microbiological Properties, 2nd edn (American Society of Agronomy, 1982).
18. Robson, D. E. Gas flux. In Klute, A, Methods of Soil Analysis, Part 1, Physical and Mineralogical Methods. Agronomy Monographs, 2nd ed, Vol. 9 (American Society of Agronomy, 1986).
19. Xie, F. et al. Response of N₂O emission to manure application in field trials of agricultural soils across the globe. Sci. Total Environ. 733, 139390 (2020).
20. Caruana, M. L. et al. Biochar’s role in mitigating soil nitrous oxide emissions: A review and meta-analysis. Agric. Ecosyst. Environ. 191, 5–16 (2014).
21. Chen, Z. et al. Nitrous oxide emissions from cultivated black soil: A case study in Northeast China and global estimates using empirical model. Glob. Biogeochem. Cycl. 28(11), 1311–1326 (2014).
22. Hayakawa, A. et al. N₂O and NO emissions from an Andisol field as influenced by pelleted poultry manure. Soil Biol. Biochem. 41(3), 521–529 (2009).
23. Gergorich, E. G. et al. Greenhouse gas contributions of agricultural soils and potential mitigation practices in Eastern Canada. Soil Till. Res. 83(1), 53–72 (2005).
24. Mosier, A. R. et al. Net global warming potential and greenhouse gas intensity in irrigated cropping systems in Northeastern Colorado. J. Environ. Qual. 35(4), 1584–1589 (2006).
25. Li, J. et al. Soil labile organic carbon fractions and soil organic carbon stocks as affected by long-term organic and mineral fertilization regimes in the North China Plain. Soil Till. Res. 175, 281–290 (2018).
26. Laurent, C. et al. Increased soil pH and dissolved organic matter after a decade of organic fertilizer application mitigates copper and zinc availability despite contamination. Sci. Total Environ. 709, 135927 (2020).
27. van Groenigen, J. W. et al. Nitrous oxide emissions from urine-treated soil as influenced by urine composition and soil physical conditions. Soil Biol. Biochem. 37(3), 463–473 (2005).
28. Chang, N. et al. Impacts of nitrogen management and organic matter application on nitrous oxide emissions and soil organic carbon from spring maize soils in the North China Plain. Soil Till. Res. 196, 104441 (2020).
29. Kuroiwa, M. et al. Gross nitrification rates in four Japanese forest soils: Heterotrophic versus autotrophic and the regulation factors for the nitrification. J. For. Res. 16(5), 363–373 (2011).
30. Iw, E. et al. Crop yield and N₂O emission affected by long-term organic manure substitution fertilizer under winter wheat-summer maize cropping system. Sci. Total Environ. 732, 139321 (2020).
31. Migliorati, M. D. A. et al. Influence of different nitrogen rates and DMPP nitrification inhibitor on annual N₂O emissions from a subtropical wheat–maize cropping system. Agric. Ecosyst. Environ. 186, 33–43 (2014).
32. Huerfano, X. et al. DMPSA and DMPP equally reduce N₂O emissions from a maize-ryegrass forage rotation under Atlantic climate conditions. Atmos. Environ. 187, 255–265 (2018).
33. Akiyama, H., Yan, X. & Yagi, K. Evaluation of effectiveness of enhanced-efficiency fertilizers as mitigation options for N₂O and NO emissions from agricultural soils: Meta-analysis. Glob. Change Biol. 16(6), 1857–1864 (2010).
34. Qiao, C. et al. How inhibiting nitrification affects nitrogen cycle and reduces environmental impacts of anthropogenic nitrogen input. Glob. Change Biol. 21(3), 1249–1257 (2015).
35. Martins, M. R. et al. Strategies for the use of urease and nitrification inhibitors with urea: Impact on N₂O and NH₃ emissions, fertilizer-15N recovery and maize yield in a tropical soil. Agric. Ecosyst. Environ. 247, 54–62 (2017).
36. Lam, S. K. et al. Using nitrification inhibitors to mitigate agricultural N₂O emission: A double-edged sword?. Glob. Change Biol. 23(2), 485–489 (2017).
37. Mariano, E. et al. Effects of N application rate and dicyandiamide on the fate of ¹⁵N fertilizer and the abundance of microbial genes in a sandy soil amended with sugarcane litter. J. Soil Sci. Plant Nutr. 22(1), 359–373 (2022).
38. de Paulo, E. N. et al. Nitrification inhibitor 3,4-Dimethylpyrazole phosphate improves nitrogen recovery and accumulation in cotton plants by reducing NO₃ leaching under ¹⁵N-urea fertilization. Plant Soil 469(1), 259–272 (2021).
39. Li, H. et al. Chemical fertilizers could be completely replaced by manure to maintain high maize yield and soil organic carbon (SOC) when SOC reaches a threshold in the Northeast China Plain. J. Integr. Agric. 16(4), 937–946 (2017).
40. Ren, F. et al. Spatial changes and driving variables of topsoil organic carbon stocks in Chinese croplands under different fertilization strategies. Sci. Total Environ. 767, 144350 (2021).
41. Zhang, W. et al. Soil organic carbon dynamics under long-term fertilizations in arable land of northern China. Biogeoosciences 7(2), 409–425 (2010).
42. Ding, X. et al. Changes in soil organic carbon pools after 10 years of continuous manuring combined with chemical fertilizer in a Mollisol in China. Soil Till. Res. 122, 36–41 (2012).
43. Singh, B. R. & Lal, R. The potential of soil sequestration through improved manuring practices in Norway. Environ. Dev. Sustain. 7(1), 161–184 (2005).
44. Zhang, W. et al. Relative contribution of maize and external manure amendment to soil carbon sequestration in a long-term intensive maize cropping system. Sci. Rep. 5(1), 10791 (2015).
45. Li, H. et al. Chemical fertilizers could be completely replaced by manure to maintain high maize yield and soil organic carbon (SOC) when SOC reaches a threshold in the Northeast China Plain. J. Integr. Agric. 16(4), 937–946 (2017).

Acknowledgements
This study was financially supported by the Non-Profit Research Foundation for Agriculture (201103039), Open Research Project of Shouguang Facilities Agriculture Center in Institute of Applied Ecology (2018SG-S-02) and the National Natural Science Foundation of China (41701300), the Natural Science Research Programme of Huai’an (HAB202055) and Huai’an Excellent Youth Science Foundation.

Author contributions
Conceptualization, W.Y. and H.X.; Investigation, S.K. and D.D.; writing—original draft preparation, D.D.; writing—review and editing, W.Y. and H.S.; visualization, D.D. and S.K.; supervision, W.Y.; funding acquisition, W.Y., H.X. and D.D. All authors have read and agreed to the published version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-19592-9.

Correspondence and requests for materials should be addressed to H.X.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022