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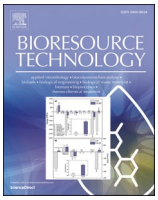

Addition of cellulose and hemicellulose degrading microorganisms intensified nitrous oxide emission during composting

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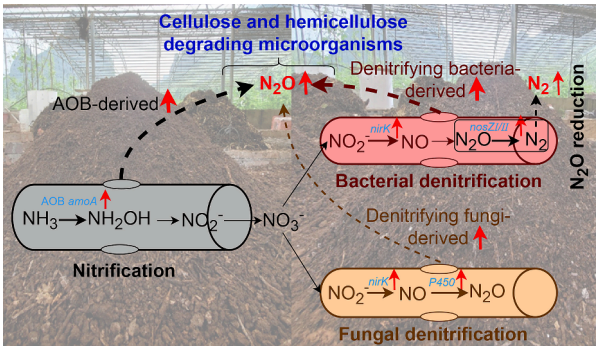
HIGHLIGHTS

- Composting with cellulose and hemicellulose-degrading microorganisms inoculated.
- Microbial inoculation promoted AOB and denitrifying bacteria derived N_2O production.
- Microbial inoculation increased AOB *amoA* and denitrifying *nirK* gene abundances.
- Microbial inoculation enhanced N_2O reduction to N_2 by increasing *nosZ*I and *nosZ*II.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims to clarify the mechanisms underlying effects of inoculating cellulose and hemicellulose-degrading microorganisms on nitrous oxide (N_2O) emissions during composting with silkworm excrement and mulberry branches. Inoculation with cellulose and hemicellulose–degrading microorganisms resulted in significant increases of total N_2O emission by 10.4 ± 2.0 % (349.1 ± 6.2 mg N kg^{-1} dw) and 26.7 ± 2.1 % (400.6 ± 6.8 mg N kg^{-1} dw), respectively, compared to the control (316.3 ± 3.6 mg N kg^{-1} dw). The stimulation of N_2O emission was attributed to the enhanced contribution of ammonia-oxidizing bacteria (AOB) and denitrifying bacteria to N_2O production, as evidenced by the increased AOB *amoA* and denitrifying *nirK* gene abundances. Moreover, microbial inoculation stimulated N_2O reduction to N_2 owing to increased abundances of *nosZ*I and *nosZ*II genes. These findings highlight the necessity to develop cost-effective and environmentally friendly strategies to reduce N_2O emissions when cellulose and hemicellulose-degrading microorganisms are inoculated during composting.

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1. Introduction

It's anticipated that annual solid waste production will skyrocket by 70 % by 2050 relative to 2016, reaching 3.4 billion tons, of which around 46 % is organic wastes (Awasthi et al., 2018). Composting represents an effective strategy for converting a portion of organic wastes into organic fertilizers (Zhao et al., 2022). Nevertheless, composting is a prominent contributor to atmospheric nitrous oxide (N_2O) (Nordahl et al., 2023; Yang et al., 2019). It is estimated that 0.02–9.9 % of the initial N would be lost as N_2O during composting (Pardo et al., 2015). Given that N_2O is a potent greenhouse gas in the troposphere and is a primary agent of ozone layer depletion in the stratosphere in the 21st century (Nisbet et al., 2021), it is urgently needed to reveal the key processes responsible for N_2O production to facilitate identifying strategies to effectively suppress N_2O emissions during composting.

Inoculation with exogenous microorganisms has been demonstrated to bolster the microbial population, alter microbial structure and metabolism, and thus expedite the degradation of organic material, thereby improving composting efficiency (Hoang et al., 2022; Huang et al., 2022; Shan et al., 2021). Inoculation with lignocellulose-degrading microorganisms has been found to accelerate the conversion of organic matter via influencing the temperature, oxygen content, carbon (C), and mineral N concentration throughout the composting process (Wang et al., 2023a; Wang et al., 2022; Yu et al., 2023). As temperature, oxygen content, carbon (C), and mineral N concentration are factors which usually affect N transformations and their related microbial communities or N_2O production (Hoang et al., 2022), inoculation with lignocellulose-degrading microorganisms may subsequently influence N transformations or N_2O production during composting. For instance, Yu et al. (2020) found that inoculation with lignocellulose-degrading microorganisms mitigated N_2O emission by decreasing the *nirS*, *nirK*, and *nosZ* gene abundances that involved in denitrification due to the decreased supply of nitrate (NO_3^-) during chicken manure and wheat straw composting. One limitation, however, is that most studies have predominantly focused on composting efficiency, but ignored N_2O emissions and the underlying mechanisms during composting.

Biogenic N_2O production is primarily driven by nitrifying and denitrifying microorganisms during N cycling processes (Huang et al., 2022; Lv et al., 2020). Autotrophic nitrification, which is also the rate-limiting step of N cycling, is the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-). This process is driven by ammonia-oxidizing bacteria (AOB), archaea (AOA), and complete ammonia oxidizers (Comammox) (Lehtovirta-Morley, 2018). Nitrifier and denitrifier-induced denitrification, including nitrifer denitrifcation, bacteria denitrification, and fungal denitrification, have been found to be the main pathways for N_2O production during composting (Cao et al., 2021; Maeda et al., 2017). It is important to note that fungal denitrification diverges from bacterial denitrification as fungi lack N_2O reductase, positioning them as a potentially significant source of N_2O (Mothapo et al., 2015). Despite the presence of multiple N_2O production pathways, there exists only a single biogenic N_2O sink in the biosphere, i.e., the reduction of N_2O to N_2 by denitrifying or non-denitrifying microorganisms that possess *nosZ*I and *nosZ*II genes, respectively, encoding the N_2O reductase (Hallin et al., 2018). Understanding the source of N_2O is crucial for unraveling the mechanisms underlying N_2O production during composting. Therefore, it is crucial to integrate multiple methods, such as isotopomer analysis, molecular assays, and inhibitor techniques to quantitate the contribution of the major pathways to N_2O production at different phases of composting. Nevertheless, very limited studies have been conducted to investigate the contribution of multiple production pathways to N_2O production during composting (Cao et al., 2018; Yang et al., 2023). Among the limited studies, Cao et al. (2021) showed that nitrifier denitrification dominates N_2O production; whereas Yang et al. (2023) reveled that nitrification and/or fungal denitrification is responsible for N_2O production during composting. These contradictory results suggest that the understanding of N_2O production is insufficient during composting.

In the current study, N_2O emission rates and the related production pathways were investigated during the composting with silkworm excrement and mulberry branches with or without the inoculation of cellulose and hemicellulose degrading microorganisms. Inoculation with lignocellulose-degrading microorganisms has been demonstrated to enhance lignocellulose degradation, leading to an anaerobic conditions (Yu et al., 2023), which benefit denitrifying microorganisms. In light of this, it was hypothesized that the addition of cellulose and hemicellulose degrading strains would potentially increase N_2O production via denitrification.

2. Materials and methods

2.1. Experimental composting and sample collection

The composting experiment was conducted at Huanjiang Observation and Research Station for Karst Ecosystems, the Chinese Academy of Sciences. Silkworm excrement and mulberry branches, provided by local farmers, were used as composting materials with the branches being cut into fragments smaller than 1 cm. The starting compost had C:N ratios between 25 and 28. Based on the C:N ratios of individual raw materials, the silkworm excrement was thoroughly mixed with the mulberry fragments in a weight-to-weight ratio of 9:1 for the composting (see supplementary materials).

The phylogenetic relationships of microbial strains that degrade cellulose and hemicellulose are presented in the Supplementary Materials (see supplementary materials). Through gene sequencing, the optimal combination of cellulose-degrading microbes was found to be *Bacillus cereus ZXX*, *Bjerkandera adusta strain ZX6*, *Trichoderma harziamm ZXL*, *Cladosporium* sp. *strian ZXY* and *Cladosporium* sp. *strian ZBX*. The optimal combination for hemicellulose-degrading microbes was found to be *Cladosporium cladosporides strian BZA1*, *Cladosporium* sp. *BZQ*, *Cladosporium* sp. *BZ10*, *Trametes* sp. *BZ22* and *Bacillus licheniformis strain BX6*.

Three treatments were included, i.e., SM (control, 900 kg silkworm excrement + 100 kg mulberry branch), SMC (900 kg silkworm excrement + 100 kg mulberry branch + 1 % (V/W) cellulose degrading strains) and SMHC (100 kg silkworm excrement + 100 kg mulberry branch + 1 % (V/W) hemicellulose degrading strains). The concentration of functional microbial inoculum was 1.0×10^8 CFU mL^{-1} for both SMC and SMHC treatments. The compost's initial moisture content was set between 55 and 60 %, with no subsequent adjustment during the composting process. The initial size of each compost pile was 1.0 m (diameter) × 0.8 m (height) and was replicated thrice. The composting period was divided into the initial phase (IPP), mesophilic phase (MEP), thermophilic phase (THP), and mature phase (MAP) according to the variation of composting temperature. The compost piles were turned over every 3 days. The temperature for SM, SMC and SMHC treatments at the initial (day 1), mesophilic (day 2 to 3), thermophilic (day 4 to 16) and mature (day 17 to 60) phases is presented in the supplementary materials (see supplementary materials).

Sampling was conducted at day 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60. Samples were taken from various positions within each compost pile, then mixed to create a composite sample for each compost. Each composite sample was divided into three parts. The first part was air-dried and used to measure pH, electrical conductivity and total nitrogen (TN). The second part was stored at 4 °C for incubation experiments or for determining dissolved organic carbon (DOC), dissolved organic nitrogen (DON), NH_4^+ and NO_3^-. The third part was stored at −80 °C, and used for DNA extraction and molecular analysis.

2.2. Determination of composting properties

The pH was determined using a Mettler Toledo FE28 pH meter (FE28, Mettler Toledo, Switzerland), while electrical conductivity was

determined with a DDSJ-308A conductivity meter from LEICI (DDSJ-308A, LEICI, Japan) (Liu et al., 2023). DOC concentration was measured using wet oxidation method, whereas persulfate oxidation method was utilized for DON determination (Duan et al., 2019). An autoanalyzer (FIAstar 5000, FOSS, Sweden) was employed to measure the concentrations of NH_4^+ and NO_3^-. TN was assayed by an Elementar analyzer (Elementar Analysesysteme, Hanau, Germany).

2.3. Determination of gas flux and isotopic signatures of nitrous oxide

In each compost pile, three static PVC chambers were installed to measure fluxes of carbon dioxide (CO_2). Measurements were taken three times weekly at the phases of IPP, MEP and THP, and twice weekly at MAP. Each chamber comprised an anchor ring (25 cm in diameter and 10 cm in height), and a removable cover (25 cm in diameter and 35 cm in height). Samples were taken between 8:00 and 11:00 am at each sampling date. Gas samples, 50 mL each, were collected at intervals of 0, 10, 20, and 30 min after the chamber was closed. A 60 mL plastic syringe was used for the collection, and the samples were immediately transferred to a 120 mL Teflon FEP air bag (Teflon® FEP; Delin® Co. Ltd., China). Concentrations of CO_2 and N_2O were subsequently measured using an Agilent GC 7890A gas chromatograph (Agilent, USA). The CO_2 and N_2O emission rate (mg C or N kg^{-1} dry weight d^{-1}) was calculated according to equation. (1):

$$Flux = \frac{V}{m} \times \frac{\triangle c}{\triangle t} \times M \times \frac{273}{273 + T} \times 22.4 \quad (1)$$

where $\Delta c/\Delta t$ is the change in gas concentration inside the chamber over time; M is the molar mass of CO_2 or N_2O; T is the temperature in the chamber; V is the headspace air volume in the static PVC chamber and m is the dry weight of composting material.

Samples for the analysis of N_2O isotopomer ratios ($\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ and $\delta^{18}O$) were gathered at day 1, 3, 8, and 30 during the composting process, representing the four distinct composting phases. The ratios were determined using the G5131-i Isotope and Gas Concentration Analyzer (Picarro Inc., Santa Clara, CA). The site preference (SP) was obtained by subtracting $\delta^{15}N^{\beta}$ from $\delta^{15}N^{\alpha}$. N_2O production and reduction were evaluated using the isotopocule mapping approach (Buchen et al., 2018; Duan et al., 2019), including nitrifier nitrification/fungal denitrification and heterotrophic denitrification/nitrifier denitrification. Two scenarios were considered. In scenario a, N_2O is produced and then reduced by denitrifiers before mixing with N_2O generated from nitrification. In scenario b, N_2O is produced from nitrification and denitrification processes, mixed, and subsequently reduced. As there was no rainfall during composting, and groundwater was used to adjust compost moisture content, the $\delta^{18}O$–H_2O was not measured. Instead, a $\delta^{18}O$–H_2O value of −6.0 ‰ was adopted to correct the $\delta^{18}O$ value of N_2O ($\delta^{18}O_{N2O/H2O}$).

2.4. Selective inhibition experiment

Compost samples from day 1, 3, 8 and 30 were used in the incubation experiment, which was aimed to determine N_2O production by AOA, AOB, Comammox, fungal and bacterial dominated processes using the selective inhibition technique according to protocols described elsewhere (Maeda et al., 2017; Sun et al., 2022). To revive microbial activity, fresh compost samples (equivalent to 5 g dry weight) were placed in 100 mL serum bottles and incubated at 25 °C for a duration of 5 days. Subsequently, three replicate microcosms were prepared for each of the following nine treatments. To measure the contribution of ammonia oxidizers to N_2O production, five treatments were applied, i.e., (1) NH_4Cl; (2) NH_4Cl + 300 μM PTIO (AOA inhibitor); (3) NH_4Cl + 0.03 % (v/v) 1-octyne (AOB inhibitor); (4) NH_4Cl + 100 μM chlorate (Comammox inhibitor), (5) NH_4Cl + 0.01 % (v/v) acetylene (ammonia oxidizers inhibitor). To investigate the contribution of denitrifying fungi and bacteria to N_2O production, another four treatments were applied, i.e., (6) KNO_3, (7) KNO_3 + 10.0 mg g^{-1} cycloheximide (fungi inhibitor), (8) KNO_3 + 5.0 mg g^{-1} streptomycin (bacteria inhibitor), and (9) KNO_3 + 10.0 mg g^{-1} cycloheximide + 5.0 mg g^{-1} streptomycin (fungi and bacteria inhibitor).

Every serum bottle was sealed with a butyl rubber stopper and further closed with aluminum. Following a 48-hour incubation at 25 °C, 30 mL of gas from the headspace of each flask was collected using an injection syringe. The N_2O concentration was then analyzed with a gas chromatograph (Agilent GC 7890A, Agilent, USA). As previously reported (Maeda et al., 2017; Sun et al., 2022), N_2O production by AOA, AOB, Comammox, fungi, or bacteria was calculated as the difference between the N_2O flux in the antibiotic-free treatment and that in the antibiotic treatment. Therefore, the contribution (%) of individual pathways to total N_2O production was calculated using Equations (2–6):

$$Contribution_{AOA}(\%) = \frac{N_2O_{NH_4^+} - N_2O_{ACE} - N_2O_{PTIO}}{N_2O_{NH_4^+}} \quad (2)$$

$$Contribution_{AOB}(\%) = \frac{N_2O_{NH_4^+} - N_2O_{ACE} - N_2O_{OCT}}{N_2O_{NH_4^+}} \quad (3)$$

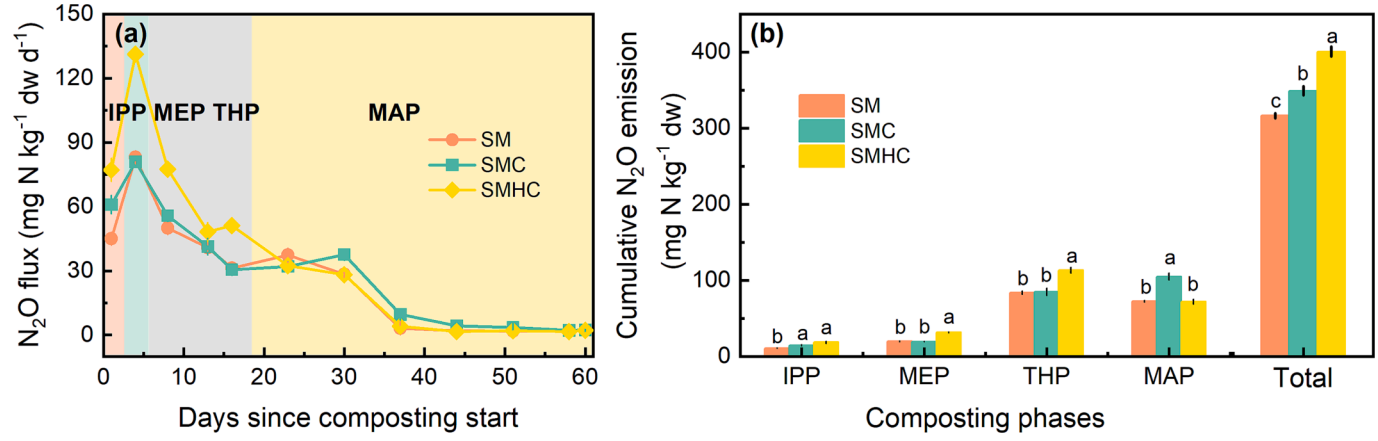

Fig. 1. Effects of inoculation with cellulose and hemicellulose degrading microorganisms on N_2O flux and cumulative N_2O emission during composting. SM: composting of silkworm excrement and mulberry branches; SMC: composting of silkworm excrement and mulberry branches with cellulose degrading microorganisms' inoculation; SMHC: composting of silkworm excrement and mulberry branches with hemicellulose degrading microorganisms' inoculation, respectively. IPP, MEP, THP and MAP represent the initial, mesophilic, thermophilic, and mature phases of composting, respectively. Values are presented as means ± standard deviations (n = 3). Different letters represent significant difference at $p < 0.05$ among treatments.

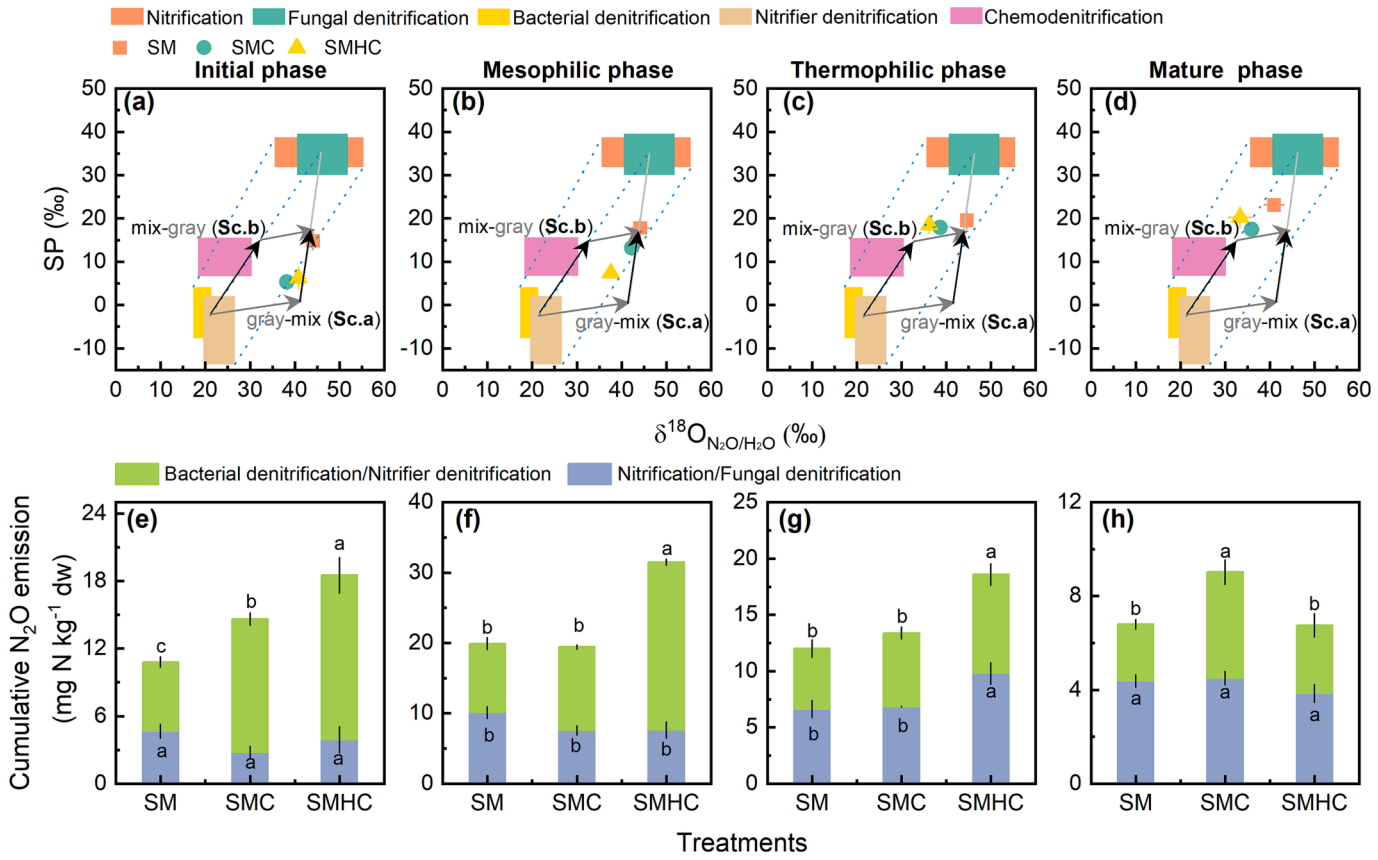

Fig. 2. The (a–d) isotopocule values of compost–emitted N_2O plotted per treatment in the isotopocule map (SP and $\delta^{18}O_{N2O/H2O}$ values) and (e–h) modeled source partitioning (nitrification/fungal denitrification vs bacterial denitrification/nitrifier denitrification) at the initial (a, e), mesophilic (b, f), thermophilic (c, g), and mature phases (d, h) of composting. SM: composting of silkworm excrement and mulberry branches; SMC: composting of silkworm excrement and mulberry branches with cellulose degrading microorganisms' inoculation; SMHC: composting of silkworm excrement and mulberry branches with hemicellulose degrading microorganisms' inoculation, respectively. Values are presented as means ± standard deviations (n = 3). Different letters represent significant difference at $p < 0.05$ among treatments.

$$Contribution_{Comammox}(\%) = \frac{N_2O_{NH_4^+} - N_2O_{ACE} - N_2O_{chlorate}}{N_2O_{NH_4^+}} \quad (4)$$

$$Contribution_{bacteria}(\%) = \frac{N_2O_{NO_3^-} - N_2O_{str}}{N_2O_{NO_3^-} - N_2O_{cyc+str}} \quad (5)$$

$$Contribution_{fungi}(\%) = \frac{N_2O_{NO_3^-} - N_2O_{cyc}}{N_2O_{NO_3^-} - N_2O_{cyc+str}} \quad (6)$$

2.5. DNA extraction and quantitative real-time PCR (qPCR)

Compost samples collected at day 1, 3, 8 and 30 were used to quantify the abundances of N_2O-related functional genes. Compost samples (0.25 g) were subjected to DNA extraction with DNeasy® Powersoil® Pro Kits following the manufacturer's instructions (Qiagen, Benelux BV, Germany). The quality and quantity of DNA were assessed using a NanoDrop 2000 (NanoDrop Technologies, DE, USA). Subsequently, the functional genes AOA, AOB, Comammox *amoA*, and *nirK*, *nirS*, fungal *nirK*, *P450*, *nosZI* and *nosZII* were quantified on a Lightcycler 96 system (Roche, Basel, Switzerland) with triplicate samples of the diluted DNA at a concentration of 10 ng μL^{-1}. Each plate included triplicate samples of purified plasmid standards and negative controls. The amplification efficiencies for these genes ranged from 89.5 % to 94.3 %. The primers, and sequences of the genes were analyzed following the procedures described by Duan et al. (2019).

2.6. Statistical analyses

Before statistical analyses, Shapiro-Wilk test was used to examine data normality, and data were logarithmically transformed when necessary. Significant differences in compost properties, cumulative N_2O and CO_2 emissions, the contributions of functional microbial groups to N_2O production, and the abundances of functional genes among treatments were examined using one-way ANOVA with Tukey's post hoc test, with significance set at $p < 0.05$. These statistical analyses were performed with SPSS 20.0 (IBM Co., Armonk, NY, USA).

3. Results and discussion

3.1. Effect of microbial inoculation on nitrous oxide emissions

N_2O emission peaked at the mesophilic phase, and then decreased through the mature phase (Fig. 1a). It is well established that temperature has a profound effect on microbial community diversity and activity with higher temperatures stimulating microbial activity, and enhancing organic matter degradation as well (Wang et al., 2023b). At the mesophilic phase, rapid degradation of organic compounds occurs owing to the highest temperature, and in turn promotes NH_4^+ production via ammonification (Hoang et al., 2022). In the current study, a parallel increase in temperature and DOC concentration was observed, coinciding with heightened NH_4^+ production and an enhanced N_2O emission rate at the mesophilic phase, suggesting that increased temperature stimulated organic matter degradation, NH_4^+ production and subsequently N_2O production (see supplementary materials). The increased

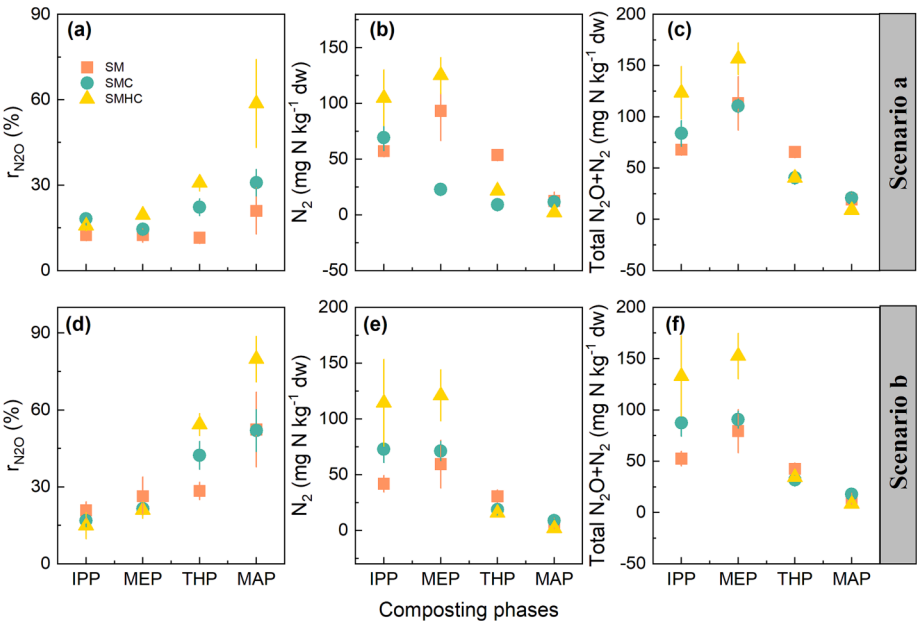

Fig. 3. Effects of inoculation with cellulose and hemicellulose degrading microorganisms on modeled residual, unreduced N_2O fraction (r_{N2O}) and the N_2 and total N_2O + N_2 fluxes for the scenarios (a and b) during composting. SM: composting of silkworm excrement and mulberry branches; SMC: composting of silkworm excrement and mulberry branches with cellulose degrading microorganisms' inoculation; SMHC: composting of silkworm excrement and mulberry branches with hemicellulose degrading microorganisms' inoculation, respectively. IPP, MEP, THP and MAP represent the initial, mesophilic, thermophilic, and mature phases of composting, respectively. Values are presented as means ± standard deviations (n = 3). Different letters represent significant difference at $p < 0.05$ among treatments.

N_2O emission in the current study could be due to the stimulated nitrification and/or denitrification from intermediate product, such as hydroxylamine and nitrite obtained by the oxidation of NH_4^+ by ammonia monooxygenase enzyme (Li et al., 2022; Manu et al., 2021).

The cumulative N_2O emissions from SMC and SMHC were 10.4 ± 2.0 % and 26.7 ± 2.1 % higher compared with SM, respectively (Fig. 1a, b). N_2O is predominantly generated from incomplete nitrification and denitrification, with nitrifiers converting NH_4^+ into NO_3^-, and denitrifiers subsequently reducing NO_3^- to gaseous N compounds, including N_2O (Yasmin et al., 2022). In the current study, the increase in N_2O emissions was concomitant with the increase in NH_4^+ and with the decrease in NO_3^- concentration under SMC and SMHC treatments, indicating that inoculation with cellulose and hemicellulose-degrading microorganisms likely promoted the transformation of NO_3^- to N_2O, and the production of N_2O from denitrification (see supplementary materials). The inoculation of cellulose and hemicellulose-degrading microorganisms into compost amplified microbial activity, and subsequently increased oxygen consumption by these microbes, resulting in elevated emissions of CO_2 (Greff et al., 2022; Yu et al., 2023). Both the SMC and SMHC treatments resulted in increased CO_2 emissions (see supplementary materials). Consequently, the limited availability of oxygen weakens the competition between nitrifiers and denitrifiers (Cao et al., 2021), and thereby increases the contribution of denitrification in N_2O production. Furthermore, high concentrations of DOC are recognized to create anaerobic microsites by stimulating heterotrophic microbial activity, thereby fostering favorable conditions for heterotrophic denitrification (Grave et al., 2018). It was reported that the stimulated degradation of organic matter by inoculating microorganisms would produce more DOC (Qu et al., 2023). In the current study, SMC and SMHC treatments increased DOC concentrations, which in turn likely contributed to N_2O production from heterotrophic denitrification by increasing anaerobic microsites (see supplementary materials).

3.2. Responses of nitrous oxide production and reduction to microbial inoculation

Based on the isotopocule mapping approach with SP and $\delta^{18}O$ values of N_2O (Buchen et al., 2018), N_2O samples from the SM, SMC, and SMHC treatments were found to fall within the region defined by the mixing line of bacterial denitrification/nitrifier denitrification and the N_2O reduction line at the initial and mesophilic phases (Fig. 2). This suggests that at low SP values, either heterotrophic bacteria or AOB might be significant contributors to N_2O production. However, the SP values of N_2O increased and ranged from 17.9 to 19.6 ‰ at the thermophilic phase and from 17.5 to 19.6 ‰ at the mature phase, implying that the fraction of nitrification/fungal denitrification to N_2O production increased at the two phases. For SMC and SMHC treatments, 61.0–81.2 % and 75.9–78.9 % of total N_2O emissions were contributed by bacteria denitrification/nitrifier denitrification at the initial and mesophilic phases, respectively, corroborating that bacterial denitrification/nitrifier denitrification, rather than nitrification/fungal denitrification, dominated N_2O production during the composting (Fig. 2). Excess NH_4^+ and high temperature (>40 °C) may inhibit the activities of nitrifying microorganisms at the early phases of composting (Guo et al., 2020). Moreover, during the composting process, organic matter provides C as energy for denitrification and influences oxygen availability, which in turn affects N_2O production (Cao et al., 2021). In the present study, the high CO_2 emission and NH_4^+ accumulation along with the low SP values and negligible NO_3^- production at the initial and mesophilic phases suggest that nitrifier denitrification could be a major pathway of N_2O production at the two phases (see supplementary materials). However, the decrease in NH_4^+ concentration was concurrent with an increase in NO_3^- and SP values, which synchronized with the decrease in DOC concentration and CO_2 flux, indicating that nitrifiers were active and nitrification was the prevailing pathway for N_2O production at thermophilic and mature phases.

Compared to SM treatment, microbial addition significantly increased bacteria denitrification/nitrifier denitrification derived N_2O production during composting (Fig. 2). This was presumably due to the

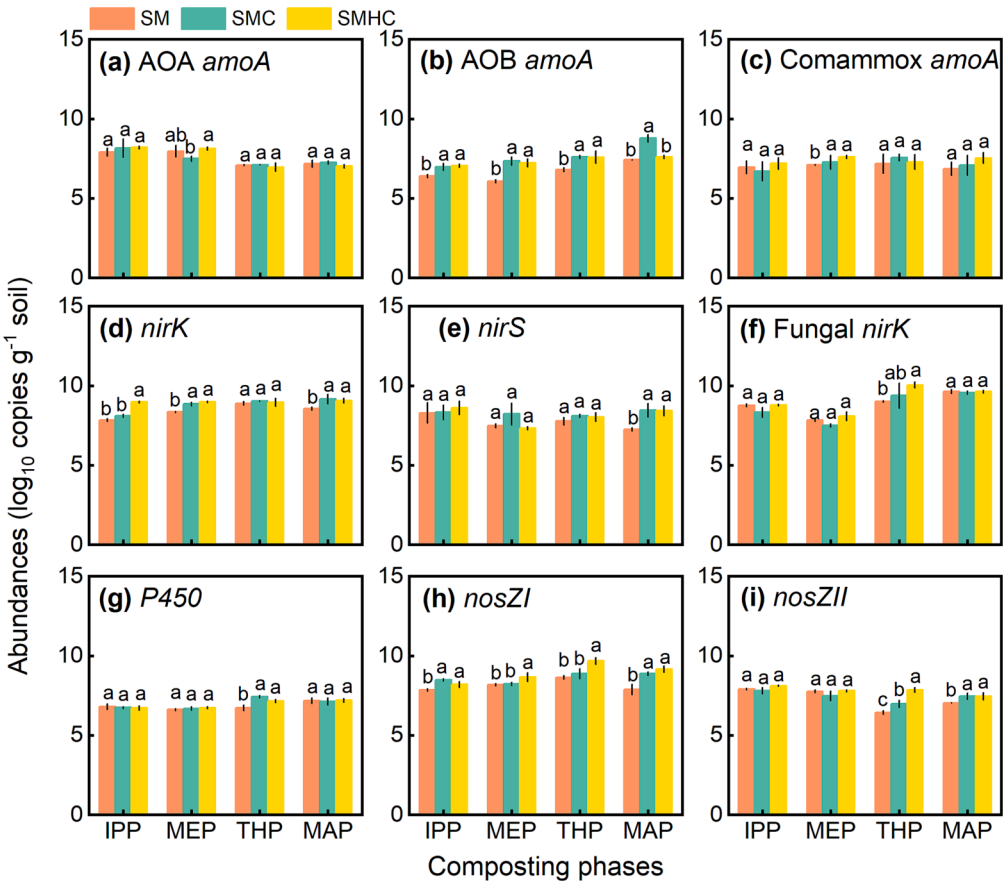

Fig. 4. Effects of inoculation with cellulose and hemicellulose degrading microorganisms on ammonia oxidizers and denitrifiers gene abundances during composting. SM: composting of silkworm excrement and mulberry branches; SMC: composting of silkworm excrement and mulberry branches with cellulose degrading microorganisms' inoculation; SMHC: composting of silkworm excrement and mulberry branches with hemicellulose degrading microorganisms' inoculation, respectively. IPP, MEP, THP and MAP represent the initial, mesophilic, thermophilic, and mature phases of composting, respectively. Values are presented as means ± standard deviations (n = 3). Different letters represent significant difference at $p < 0.05$ among treatments.

fact that the inoculated functional microorganisms accelerated the decomposition of structured substances (e.g., lignocellulose), and the metabolic activity of microorganisms, leading to the increased CO_2 emission and decreased oxygen availability (Yu et al., 2023). The anaerobic environment potentially favored the growth of denitrifiers and their contribution to N_2O production. Moreover, the accumulation of NH_4^+, along with low oxygen availability at the mature phase, could have stimulated AOB activity and nitrite production, potentially stimulating N_2O production from nitrifier denitrification (Wrage-Mönnig et al., 2018). Compared to SM, AOB *amoA* abundance was significantly increased at the initial, mesophilic and thermophilic phases under SMHC, and at all the four phases under SMC treatment (Fig. 4b). However, the abundances of AOA and comammox *amoA* were not significantly altered by microbial inoculation during the composting. These results suggest that AOB may contribute more to the transformation of NH_4^+ to NO_3^- and related N_2O production after microbial inoculation. AOB are known to thrive under anoxic conditions and exclusively possess the *nirK* gene, which is associated with nitrifier denitrification (Kuypers et al., 2018). In the current study, an increase in *nirK* gene abundance after microbial inoculation was observed at the initial, mesophilic and mature phases, indicating the potential role of AOB–driven denitrification in N_2O production (Fig. 4d). Thus, the decreased oxygen availability, and the enhanced AOB *amoA* and denitrifying *nirK* genes abundances caused by inoculated microorganisms were likely responsible for the observed increase in the contribution of nitrifier denitrification to N_2O production. In addition, the inoculated microorganisms significantly increased N_2O production from nitrification/fungal denitrification at the thermophilic phase (Fig. 2f). It has been reported that fungal denitrification is particularly benefited under conditions of high C availability (Yamamoto et al., 2017; Zhong et al., 2018). Considering that inoculation with lignocellulose-degrading microorganisms promoted the degradation of organic matter, more DOC would be accumulated (Qu et al., 2023), potentially fostering the denitrifying fungal community (Senbayram et al., 2018). In support of this, the current study revealed that inoculation with cellulose and hemicellulose-degrading microorganisms significantly increased the abundances of fungal *nirK* and *P450* genes at the thermophilic phase. Accordingly, the increased DOC concentration and abundances of fungal *nirK* and *P450* genes likely explained the increase in fungal denitrification derived N_2O production under SMHC treatment at thermophilic phase.

The total fluxes of N_2O and N_2 significantly increased by 2.3–23.3 % and 14.2–65.9 % under SMC treatment for scenarios a and b, respectively, and by 38.4–81.6 % and 92.1–152.8 %, respectively, under SMHC treatment for scenarios a and b at the initial and mesophilic phases (Fig. 3). The N_2O and N_2 emissions at the initial and mesophilic phases were attributed to complete denitrification at the anaerobic microsites caused by rapid organic matter degradation (Lu et al., 2021). Compared to SM treatment, inoculation with cellulose and hemicellulose-degrading microorganisms resulted in 16.5 % and 34.2–83.6 % higher N_2 fluxes for scenario a at the initial and mesophilic phases, respectively (Fig. 3). It has been reported that DOC:NO_3^- ratio is a predominant factor that regulates N_2O reduction with more N_2O being reduced into N_2 under conditions with higher DOC:NO_3^- ratio (Benckiser et al., 2015). In

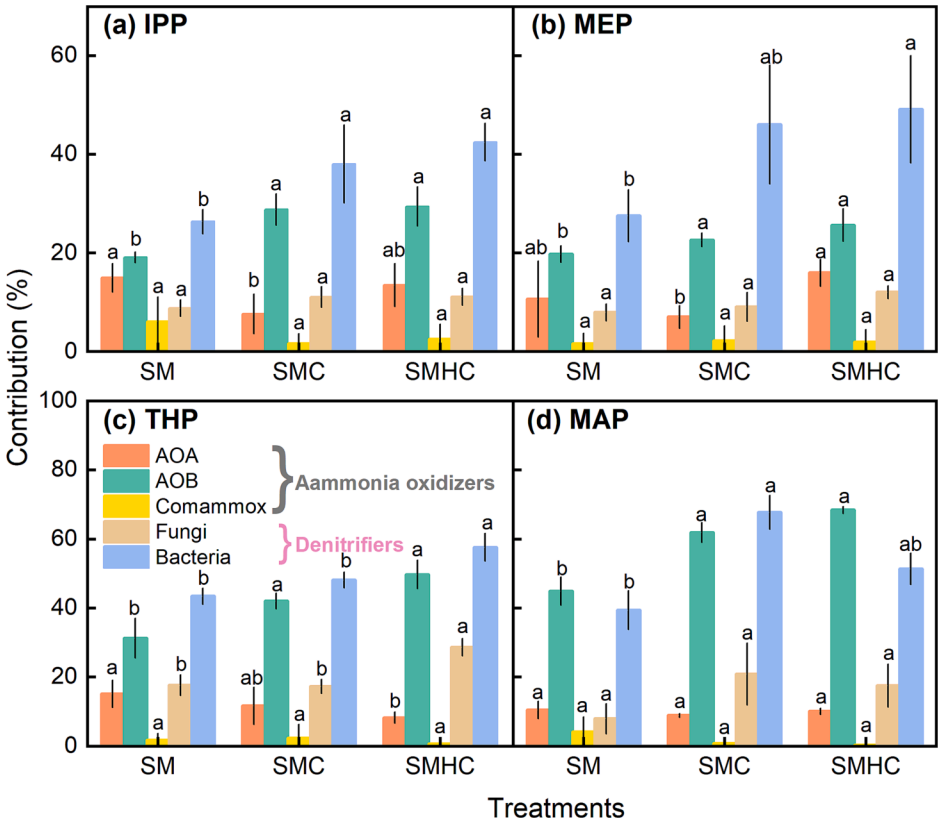

Fig. 5. Effects of inoculation with cellulose and hemicellulose degrading microorganisms on the contribution of AOA, AOB, comammox, denitrifying fungi and bacteria to N_2O production during composting. SM: composting of silkworm excrement and mulberry branches; SMC: composting of silkworm excrement and mulberry branches with cellulose degrading microorganisms' inoculation; SMHC: composting of silkworm excrement and mulberry branches with hemicellulose degrading microorganisms' inoculation, respectively. IPP, MEP, THP and MAP represent the initial, mesophilic, thermophilic, and mature phases of composting, respectively. Values are presented as means ± standard deviations (n = 3). Different letters represent significant difference at $p < 0.05$ among treatments.

the current study, DOC:NO_3^- ratio was significantly higher under SMC and SMHC treatments than the control, so that the higher DOC:NO_3^- ratio should be responsible for the greater N_2 fluxes under SMC and SMHC treatments (see supplementary materials). Additionally, SMC and SMHC significantly increased *nosZ*I gene abundances throughout the composting process, and increased *nosZ*II gene abundances at the thermophilic and mature phases (Fig. 4). Since these genes encode the enzymes that catalyze the reduction of N_2O to N_2 (Stein, 2020), their increases imply that the reduction of N_2O to N_2 should have been stimulated after microbial inoculation.

3.3. Contributions of ammonia oxidizers and denitrifying bacteria/fungi to nitrous oxide production under microbial inoculation

Ammonia-rich and alkaline environments favor the growth of AOB, whereas the growth of AOA is favored by ammonia-poor and acidic conditions (Li et al., 2023; Prosser et al., 2020). Accordingly, the higher NH_4^+ concentrations and pH under SMC and SMHC should more benefit the proliferation of AOB compared to AOA and comammox, and AOB dominated N_2O production during the nitrification process (Fig. 5). The contribution of AOB to N_2O production was increased by 14.4–50.7 % and 29.6–58.9 % respectively, under SMC and SMHC relative to the control, likely owing to the stimulated abundances of AOB *amoA* gene (Fig. 4b). Furthermore, AOB can endure anaerobic conditions and possess *nirK* gene, which is involved in nitrifier denitrification (Wrage-Mönnig et al., 2018). In the present study, *nirK* gene abundance was increased after microbial inoculation during composting, implying a potential contribution of nitrifier denitrification to N_2O production (Fig. 4d).

The relative production of N_2O from bacteria was higher than that from fungi, suggesting that bacteria were the dominant microbial group contributing to N_2O production, while fungi played a minor role, particularly at the initial and mesophilic phases (Fig. 5). This could be attributed to the conditions in the compost, as low pH and high NO_3^- levels favor fungal growth (Aldossari and Ishii, 2021). The production of N_2O from bacteria significantly ($p < 0.05$) increased by 10.8–71.7 % and 30.3–48.4 % under SMC and SMHC treatments, respectively (Fig. 5), likely due to higher abundances of the *nirK* gene associated with bacterial denitrification in microbial inoculation treatments compared to the control. Furthermore, the inoculation with hemicellulose-degrading microorganisms significantly increased the contribution of fungi to N_2O production by 62.2 % at the thermophilic phase (Fig. 5). The enhanced abundances of the fungal *nirK* and *P450* genes could explain this increase in N_2O production from denitrifying fungi under the addition of hemicellulose-degrading microorganisms, since fungal *nirK* and *p450* genes can serve as marker genes for fungal denitrification (Bösch et al., 2023; Higgins et al., 2018). The increase of anaerobic microsites resulting from intensified nitrification could have supported the stimulated proliferation of denitrifiers, leading to elevated N_2O emissions under SMHC treatment. It is therefore suggested that ventilation and turn frequency should be increased in order to decrease anaerobic microsites and in turn N_2O production via denitrification under the inoculation of cellulose and hemicellulose-degrading microorganisms during composting.

4. Conclusions

Inoculation with cellulose and hemicellulose-degrading microorganisms increased N_2O emissions during composting through increasing abundances of AOB *amoA* and denitrifying *nirK* genes, so that stimulated

contribution of AOB and denitrifying bacteria to N_2O production. Microbial inoculation enhanced N_2O emissions at the thermophilic phase via increasing the contribution of fungal denitrification to N_2O production due to increased abundances of fungal *nirK* and *p450* genes. Furthermore, microbial inoculation stimulated N_2O reduction to N_2. Taken together, the cost-effective and environmentally friendly strategies should be further developed when cellulose and hemicellulose-degrading microorganisms are inoculated during composting in order to mitigate N_2O emissions.

CRediT authorship contribution statement

Xinyi Yang: Investigation, Methodology, Writing – original draft. **Pengpeng Duan:** Writing – review & editing. **Qiumei Liu:** Investigation, Methodology. **Kelin Wang:** Writing – review & editing. **Dejun Li:** Conceptualization, Funding acquisition, Supervision, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130100.

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