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# How gut-stimulated denitrifiers influence soil $N_2O$ emission without earthworm activity

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ABSTRACT

Gut-stimulated denitrifiers are responsible for the *in vivo* emission of $N_2O$ by earthworms, and contribute to the $N_2O$ that is emitted from certain terrestrial ecosystems. To evaluate the separate effect of gut-stimulated denitrifiers on $N_2O$ emissions, earthworms or their casts were inoculated into sterilized and unsterilized soil, respectively, and compared with control (soil without inoculation) in this study, to find out if there was a difference in $N_2O$ emissions during the 15 days incubation period. Compared with the control, earthworm inoculation significantly increased $N_2O$ flux and relative densities of *nirS* and *narG* gene copy numbers both in sterilized and unsterilized soil, while no significant difference was found when we transited and recolonized those gut-stimulated denitrifiers into soil artificially. In contrast, cast inoculation did not change $CO_2$ emission in the unsterilized soil, but significant increased cumulative $CO_2$ emission in the sterilized soil. Our data suggest that the effect of gut-stimulated denitrifiers on soil $N_2O$ produce was strongly diminished when separated from earthworm activity.



Earthworms represent the major soil animal biomass in most terrestrial temperate ecosystems, and their interactions with soil microbes are thought to be an important factor on influencing the $N_2O$ balance of soils [1], although earthworms hardly produce any $N_2O$ themselves [2]. The production of $N_2O$ is linked to the microbial turnover of inorganic N by nitrifying and denitrifying organisms [3]. While the earthworm gut is a near optimal conditions for the denitrifier activity as it is essentially an anaerobic microsite where the local enrichment of mineral N, available C and favourable moisture levels [4,5]. As a result, $N_2O$ emissions from casts can be up to three times greater than from bulk soil, and gut-stimulated denitrifying bacteria may be responsible for the *in vivo* emission of $N_2O$ [2,4–8]. By inoculating earthworms into sterilized soil, from which the impacts of indigenous soil microbes are considered to be eliminated within a certain time period, Wu et al. [9] further confirmed that earthworms inoculating significantly increased $N_2O$ emissions by changing the soil bacterial community via gut-stimulated denitrifiers during the 30 days incubation, while this effect may be diminished by the competition with indigenous soil microorganisms in unsterilized soil.

However, the separate effect of gut-stimulated denitrifiers on soil $N_2O$ emission is still not clear in the existing studies, due to earthworms also indirectly influencing the production of $N_2O$ by their own activity, which usually occurs simultaneously in time and space. Thus, we inoculated earthworms or their casts into sterilized or unsterilized soil, respectively, and compared with control soil, to determine whether there were differences in soil $N_2O$ emissions during the 15 days incubation period. The aim of this study was to assess if gut-stimulated denitrifiers will increase soil $N_2O$ emission without earthworm activity.

Soil, maize straw and earthworms were collected from the same field in Wuhan, Hubei province, China. The dominant earthworm species in this field was *Metaphire guillelmi* (~70 individuals $m^{-2}$). Soil was sieved through a 5 mm mesh, well mixed, and then adjusted to 61% water-filled pore space (consistent with the field) for 7 days pre-incubation; a subset of the collected soil was then sterilized by the autoclaving method according to Wu et al. [9]. The properties of the soil at the beginning of incubation are presented in Table S1. Maize straw was oven-dried, fragmented into pieces <2 mm and then sterilized by the autoclaving method. Six earthworms (*Metaphire guillelmi*) of similar size and weight were collected, their guts were purged following the filter paper method [1], and then rinsed with sterile distilled water before using. A subset of the remaining earthworms were rinsed with sterile

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distilled water, then the fresh cast was released by gently pressing the bodies of intact worms with tweezers, from the last third to the posterior end [10]. In doing this, we assumed that the gut content corresponding to the final section of the hindgut was obtained [4]. Fresh casts were collected every day, pooled and prepared for suspension by mixing 5.2 g fresh cast with 10 mL 1/4 strength Ringer's solution [11], and then used for inoculation immediately.

The experiment in three replicates was carried out using microcosm system incubation and consisted of six treatments: sterilized/unsterilized soil inoculated with earthworms (SE/UE), fresh cast suspension (SC/UC), and a control (SCK/UCK), respectively. Sterilized or unsterilized soil (300 g) was placed in an 800 mL glass bottle. Subsequently, 1 g of maize straw was manually incorporated into the soil. Depending on the treatment, one individual adult earthworm (equivalent to ~110 individuals $m^{-2}$), or 2 mL fresh suspension was inoculated into soils at the beginning of the experiment, respectively. No earthworm or fresh suspension inoculation was set up as the counterpart control. In order to simulate the earthworm excretion process, 2 mL fresh suspension was added into the SC and UC treatments every day during the incubation. This was equivalent to the excrement amount by one individual earthworm in 24 h, which was estimated from cast production prior to commencement of experiment [12,13]. At the same time, 2 mL sterilized suspension (sterilized by the autoclaving method) was added to the earthworm inoculation and control bottles, respectively, to keep the added nutrients consistent in each treatment. All microcosms were incubated in the dark at temperatures of 26 ± 2 °C during the 15 days incubation period. Soil moisture content was manually adjusted every day by adding sterile distilled water.

Flux measurements of $N_2O$ and $CO_2$ were taken daily and determined immediately according to Baggs et al. [14] and Huang et al. [15] by gas chromatograph (GC-7890A, Agilent Technologies, USA). Fluxes were calculated assuming a linear increase in the concentration of gas over time, and the cumulative gas emissions were calculated assuming linear changes between subsequent measurements [16]. Soil was taken at day 5 and day 15 during the incubation. The quantitative PCR assay for the 16S rRNA, *narG*, *nirK*, *nirS*, and *nosZ* gene was carried out using SYBR green as the detection system according to Henry et al. [17], López-Gutiérrez et al. [18], and Kandeler et al. [19].

Statistical analyses were conducted using the SPSS 16.0 package (SPSS Inc., Chicago, IL, USA). Means (n = 3) and standard errors (SEs) were calculated. Each parameter was analyzed using a one-way analysis of variance (ANOVA) for each sampling point and least significant difference (LSD) values at the 5% level of significance ($p < 0.05$).

Soil sterilization has been widely used to identify the effects of soil organisms in the previous studies [20]. In this study, near zero soil microbial biomass carbon (MBC) values (Table S1) and $CO_2$ emission (Fig. 1B) after sterilization indicated that the impact of indigenous soil microorganism has been eliminated at the beginning of incubation. However, the delayed $CO_2$ emission in sterilized soil without inoculation may be attributed to the environmental microbial invasion (incubation was not conducted in absolutely sterile conditions) (Fig. 1B).

At the end of the experiment, all the earthworms had survived without obvious weight change in earthworm-inoculated treatments, and significantly increased $N_2O$ flux both in sterilized and unsterilized soil (Fig. 1A), which is in agreement with Wu et al. [9]. A subset of soil microorganisms ingested by earthworms, such as denitrifying and fermentative bacteria, are greatly stimulated during the gut passage, resulting in differences between casts and soils [21]. As Chapuis-Lardy et al. [22] indicated, the relative abundance of *nirK* and *nosZ* genes increased in the casts compared with the non-ingested soil. Then, these denitrifiers may transit and recolonize into the soil during the earthworm excreting, and lead to a higher $N_2O$ emission. Especially in the sterilized soil, with low

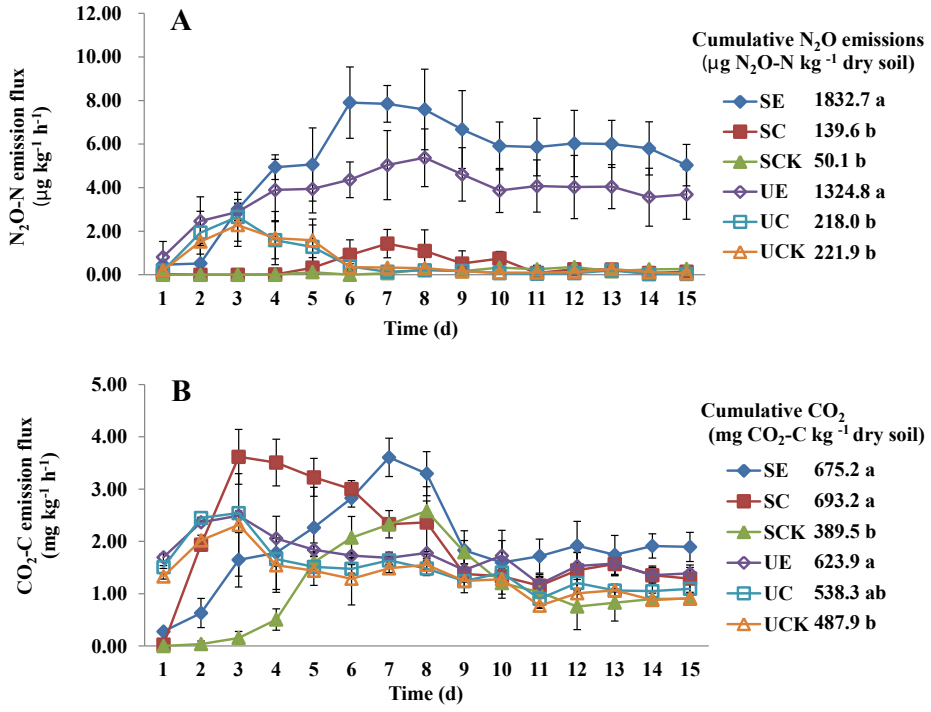


**Fig. 1.** Emission flux and cumulative emission of $N_2O$ (A) and $CO_2$ (B) from different treatments. The vertical bars in the emission flux represent the standard error. Values of cumulative emissions followed by the same lower case letter(s) are not significanty different at $P < 0.05$ in the same treated soil. SE/UE: sterilized/unsterilized with earthworms inoculation; SC/UC: sterilized/unsterilized with fresh cast suspension inoculation; SCK/UCK: sterilized/unsterilized soil without inoculation.