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Soil invertebrate fauna affect N_2O emissions from soil

IMKE KUIPER, GERLINDE B. DE DEYN, MADHAV P. THAKUR and JAN WILLEM VAN GROENIGEN

Department of Soil Quality, Wageningen University, P.O. Box 47, Droevendaalsesteeg 4, Wageningen AA 6700, The Netherlands

Abstract

Nitrous oxide (N_2O) emissions from soils contribute significantly to global warming. Mitigation of N_2O emissions is severely hampered by a lack of understanding of its main controls. Fluxes can only partly be predicted from soil abiotic factors and microbial analyses – a possible role for soil fauna has until now largely been overlooked. We studied the effect of six groups of soil invertebrate fauna and tested the hypothesis that all of them increase N_2O emissions, although to different extents. We conducted three microcosm experiments with sandy soil and hay residue. Faunal groups included in our experiments were as follows: fungal-feeding nematodes, mites, springtails, potworms, earthworms and isopods. In experiment I, involving all six faunal groups, N_2O emissions declined with earthworms and potworms from 78.4 (control) to 37.0 (earthworms) or 53.5 (potworms) mg N_2O-N m^{-2}. In experiment II, with a higher soil-to-hay ratio and mites, springtails and potworms as faunal treatments, N_2O emissions increased with potworms from 51.9 (control) to 123.5 mg N_2O-N m^{-2}. Experiment III studied the effect of potworm density; we found that higher densities of potworms accelerated the peak of the N_2O emissions by 5 days ($P < 0.001$), but the cumulative N_2O emissions remained unaffected. We propose that increased soil aeration by the soil fauna reduced N_2O emissions in experiment I, whereas in experiment II N_2O emissions were driven by increased nitrogen and carbon availability. In experiment III, higher densities of potworms accelerated nitrogen and carbon availability and N_2O emissions, but did not increase them. Overall, our data show that soil fauna can suppress, increase, delay or accelerate N_2O emissions from soil and should therefore be an integral part of future N_2O studies.

Keywords: Acari, Collembola, Enchytraeidae, greenhouse gas, Isopoda, Lumbricidae, Nematoda, nitrogen cycling, trophic interactions

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Introduction

Nitrous oxide (N_2O) plays an important role in the current debate about climate change. Its radiative forcing is, on a molar basis, 298 times stronger than carbon dioxide (CO_2) (IPCC, 2007) , and its share in global anthropogenic greenhouse gas emissions in 2004 was 7.9% (IPCC, 2007). Apart from radiative forcing, N_2O is currently the most important anthropogenic ozone–depleting compound and will probably remain so during this century (Ravishankara *et al*., 2009).

Agricultural soils emit 6.2 Tg N_2O-N yr^{-1}, primarily as the direct or indirect result of the application of mineral nitrogen (N) fertilizers (Crutzen *et al*., 2008). This corresponds to 35% of total N_2O emission (Kroeze *et al*., 1999). Nitrification, denitrification and nitrifier denitrification are the three main biochemical production pathways of N_2O from soils (Wrage *et al*., 2004; Kool *et al*., 2011). Research during the past decades has improved our understanding of N_2O dynamics in the soil and showed that its main controlling factors are as follows: the availability of N and carbon (C), soil water content (or anaerobicity), soil pH (Davidson *et al*., 2000) and temperature (Parton *et al*., 2001).

Despite this progress in N_2O research, emissions remain notoriously difficult to predict. Many models of soil N_2O emissions have been developed, ranging from the laboratory to regional or even global scale (e.g. Li, 2000; Parton *et al*., 2001). However, many knowledge gaps and challenges remain, for example, related to gaseous diffusion in soil (Chen *et al*., 2008), combining soil physics and soil biology (Blagodatsky & Smith, 2012) as well as understanding hotspots of N_2O emissions (Groffman *et al*., 2009). However, apart from the knowledge gaps mentioned by these authors, the absence of soil fauna effects in N_2O models may also contribute to their poor performance. It seems likely that such an effect exists and is substantial, given the key role of soil fauna in N mineralization and soil structural properties (Verhoef & Brussaard, 1990).

The only faunal group for which a considerable body of literature on their effects on N_2O emissions exists are earthworms. Several studies showed that earthworms can increase N_2O emissions (Tianxiang *et al*., 2008; Giannopoulos *et al*., 2010; Lubbers *et al*., 2011; Nebert

Correspondence: Jan Willem Van Groenigen, tel. +31 (0)317 484784, fax +31 (0)317 419000, e-mail: janwillem.vangroenigen@wur.nl

et al., 2011; Paul *et al*., 2012). In the drilosphere (the soil volume which is directly influenced by earthworms, including earthworm gut, casts, mucus and burrow walls), the conditions are optimal for denitrifying bacteria, resulting in local hotspots for N_2O emissions (Drake & Horn, 2006). Besides, feeding, burrowing and casting activities of earthworms affect biological, chemical and physical properties of the soil, and this can influence N_2O emissions from soil as well (Paul *et al*., 2012). A recent meta-analysis (Lubbers *et al*., 2013) found that earthworms overall significantly increase N_2O emissions by 42%.

For other soil fauna, studies on their impact on the N cycle focus on N mineralization rates rather than on N_2O emissions (Verhoef & Brussaard, 1990; de Ruiter, 1993; Bardgett & Chan, 1999; Cole *et al*., 2004; Lenoir *et al*., 2007; Osler & Sommerkorn, 2007; Kaneda & Kaneko, 2011). Soil fauna affects N mineralization by changing soil physical, chemical and biological parameters through a combination of activities. These include grazing on microflora, fragmenting organic matter, mixing organic matter into the soil, excreting nutrient-rich compounds and dispersing microbial propagules (Bardgett & Chan, 1999). In a literature study across natural and agricultural systems, Verhoef & Brussaard (1990) found a rather uniform faunal contribution to N mineralization of around 30%. Different functional groups of soil fauna, however, contribute to N mineralization differently, with largest contributions being provided by bacterial-feeding nematodes and amoeba, followed by earthworms and potworms, and minor contributions by fungal-feeding nematodes and microarthropods (de Ruiter, 1993).

All these factors affecting N mineralization can also affect N_2O dynamics and therefore suggest an effect of soil fauna on N_2O fluxes extending beyond earthworms. Earthworms not only directly affect N mineralization by feeding but also by changing the soil physical structure (van Vliet *et al*., 2004), thereby influencing diffusion rates and emission path lengths. Whether functional groups of soil fauna as distinguished in soil food webs also result in functional groups with respect to N_2O emissions remains to be tested.

This study therefore aimed to quantify and understand the effect of soil fauna on N_2O emissions from soil. In three experiments, N_2O emissions were measured in the presence of different soil invertebrate fauna species and densities. Based on knowledge about faunal contributions to N mineralization, as well as the established link between mineral N availability and N_2O emissions (Firestone *et al*., 1980), we tested three hypotheses: (i) soil invertebrate fauna other than earthworms increase soil N_2O emissions; (ii) different soil fauna species affect N_2O emissions to a different extent and (iii) the effects of soil fauna on N_2O emissions are larger with higher faunal density.

Materials and methods

In three microcosm experiments we measured N_2O emissions as affected by soil invertebrate fauna species belonging to different functional groups of soil fauna according to established soil food webs (de Ruiter, 1993). All fauna species were obtained from cultures:

1. Nematodes: The fungivorous nematode species *Aphelenchoides subtenuis* was cultured at 20 °C on *Botrytis cinerea* fungal cultures growing on PDA agar;
2. Mites: The fungivorous mite species *Acarus siro* and *Rhizoglyphus echinopus* were cultured on yeast;
3. Springtails: *Folsomia candida* was cultured on yeast and *Orchesella cincta* on wood twigs colonized with algae (*Desmococcus* sp.);
4. Isopods: *Porcellio scaber* was fed with beech leaves;
5. Potworms: *Enchytraeus albidus* was fed with composting plant material;
6. Earthworms: the epigeic compost worm *Eisenia fetida* was kept in composting horse manure.

The mites, springtails and isopods were maintained separately in Petri dishes or boxes with a bottom layer of humid plaster at 15 °C. The potworms and earthworms were maintained in microcosms with a mixture of soil and composting plant material. All fauna were cultured at 15 °C apart from the nematodes which were kept at 5 °C only during the last week prior to their use.

Experiment I

Experimental set-up. Experiment I was set-up to quantify N_2O emissions in the presence of a wide range of soil invertebrate fauna species, with hay as residue providing a C and N source. This experiment consisted of two control treatments (one with and one without hay, referred to as 'control hay' and 'control soil' respectively) and six soil invertebrate fauna treatments from all functional faunal groups which we collected. All microcosms containing fauna were amended with hay (Table 1). Fluxes of N_2O and CO_2 were measured for 61 days, after which microcosms were sampled for soil analyses and faunal extractions. The microcosms were arranged in a climate room (15 °C, 60% humidity, complete darkness) in a randomized block design, consisting of five blocks. Fluxes were measured from five replicates from the respective blocks. To enable destructive soil analyses and faunal extractions six extra replicates were set-up and distributed over three of the five blocks; three replicates were harvested at day 13 and the remaining three at day 34. The experiment therefore consisted of 8 treatments with 11 replicates, totalling 88 replicates.

The soil was loamy sand subsoil, collected from the experimental farm 'Droevendaal' (51°59′N, 5°39′E), and was heated for 20 h at 65 °C to eliminate macro and mesofauna, without losing all soil microbes (Kaneda & Kaneko, 2011). Polypropylene microcosms (diameter = 6.7 cm, height = 14 cm,

Table 1 Overview of the fauna treatments in the three experiments (Exp. I, II and III), numbers are individuals per microcosm. Hay was superficially incorporated into the topsoil, but being more diluted with soil in experiments II and III compared with experiment I

Code	Fauna	Species	Exp. I	Exp. II	Exp. III
Pw	Potworms	*Enchytraeus albidus*	50	50	50 (medium)
		E. albidus	ni	ni	10 (low)
		E. albidus	ni	ni	100 (high)
Ne	Nematodes	*Aphelenchoides subtenuis*	1100	ni	ni
Mi	Mites	*Rhizoglyphus echinopus*	200	200	ni
		Acarus siro	200	200	ni
Sp	Springtails	*Folsomia candida*	130	100	ni
		Orchesella cincta	130	ni	ni
Ew	Earthworms	*Eisenia fetida*	2	ni	ni
Is	Isopods	*Porcellio scaber*	5	ni	ni
CH	Control hay	–	–	–	–
CS	Control soil	–	–	–	–

ni, not included treatments.

Table 2 Soil parameters at the start of experiments I and II after 5 days preincubation, for control hay (CH) and control soil (CS) treatments

	NH_4	$NO_3 + NO_2$	DON	DOC	MBN	pH
CH	1.12 (±0.07)	0.71 (±0.00)	12.0 (±0.4)	22.9 (±0.1)	54.7 (±10)	6.20 (±0.02)
CS	0.00 (±0.02)	2.48 (±0.02)	2.6 (±0.2)	5.7 (±0.3)	3.9 (±0.4)	5.83 (±0.02)

Values are in mg N or C kg^{-1} dry soil, values in brackets are SEs (n = 2). DON, dissolved organic nitrogen; DOC, dissolved organic carbon; MBN, microbial biomass nitrogen.

volume = 500 cm^3) were filled with 200 g dry soil, which was first passed through a 2 mm mesh to remove stones and plant material. Distilled water was added (150 ml kg^{-1}) to reach 70% water-filled pore space (WFPS) after the soil was subsequently gently pressed until it reached a bulk density of 1.67 g cm^{-3}. The prepared microcosms were preincubated for 1 day.

Hay residue (32.8 g N kg^{-1} dry matter, 448.5 g C kg^{-1} dry matter, C : N ratio = 13.7) was chopped in pieces of 0.5–1 cm and subsequently wet autoclaved. To represent an N fertilization rate of 125 kg N ha^{-1}, 2.8 g wet hay (1.34 g of dry hay equivalent) was mixed with 30 g dry soil and distilled water (150 ml kg^{-1}). This hay–soil mixture was added on top of the soil layer of the preincubated microcosms so that each microcosm contained 230 g of dry soil equivalent and 1.34 g of dry hay. Preincubation continued for 5 days, to allow the microflora to colonize the hay–soil mixture and to reach steady-state gas emissions, which were occasionally checked.

After preincubation, soil samples were taken for baseline soil analysis (Table 2) and the experiment was started by introducing the fauna species to the microcosms. Densities corresponded to realistic field densities as reported by de Ruiter *et al*. (1995) (Table 1). Nematodes were added in suspension in 2 ml of water which were obtained by extracting the nematodes from the agar plates over a filter through which the nematodes actively crawled into tap water. For practical reasons, mites were added together with some yeast flakes from their cultures; their numbers were estimated by counting under binoculars. Earthworms were prepared according to the wet filter paper method, 48 h before addition to clean their guts (Dalby *et al*., 1996). Added springtails, potworms, earthworms and isopods were adults or large juveniles and were added individually by hand with tweezers or a brush. Yeast flakes and two drops of each nematode and potworm supernatant (not containing nematodes or potworms) of the inoculum were added to each treatment to minimize potential differences between treatments in terms of the microorganisms that may have been present in the inocula.

The microcosms were covered individually with a tightly woven black cloth to allow gas exchange, but simultaneously minimize moisture loss and prevent fauna from escaping. A large black polyethylene cloth covered all microcosms to ensure darkness. To maintain 70% WFPS, the microcosms were weighed after each flux measurement and resupplied with distilled water by gentle spraying.

N_2O and CO_2 fluxes. After fauna addition, N_2O and CO_2 fluxes were measured daily during the first week, and approximately three times a week during the remainder of the experiment. A static closed chamber technique was used to measure N_2O and CO_2 fluxes with a photoacoustic infrared multigas analyser (Type 1302; Brüel and Kjaer, Nærum, Denmark), following Velthof *et al*. (2002). Microcosms were closed with lids equipped with two rubber septa for 60 min, after which N_2O accumulation was measured. Microcosms were subsequently opened for at least 30 min, and then closed again for the CO_2

measurements. The measurements of CO_2 were conducted in a similar way as the N_2O measurements, with the exception that during N_2O measurements a soda-lime filter which was used to minimize interference effects of CO_2.

Soil analyses. Destructive soil analyses were performed on days 13 and 34, and after the final sampling on day 62. Each microcosm was divided into two halves, one of which was used for faunal extractions and the other half was used for soil analyses. After thorough mixing of the lower soil layer with the upper hay–soil mixture, a subsample was dried at 40 °C for 48 h and analysed for dissolved organic carbon (DOC) and pH ($CaCl_2$ extraction) (Houba *et al.*, 2000). Another subsample was used to determine microbial biomass nitrogen (MBN), following the chloroform fumigation and extraction technique (k_{EC} = 0.54) (Brookes & Joergensen, 2006). Subsequently, total dissolved N (Nts), ammonia (NH_4) and nitrate + nitrite ($NO_3 + NO_2$) concentrations were measured colorimetrically. To calculate the dissolved organic nitrogen (DON) content, $NH_4 + (NO_3 + NO_2)$ was subtracted from Nts.

Fauna abundances. Before soil sampling, surface dwelling *O. cincta* springtails were collected with a sucking device, and isopods and earthworms were collected by hand from the whole microcosms. Other fauna species were extracted from one half of the microcosm, and different extraction techniques were used for the different faunal groups. Nematodes were extracted with an Oostenbrink funnel (using water flow rates of 1200 ml min^{-1} before and 800 ml min^{-1} after addition of the soil sample) (Oostenbrink, 1960). The extracts were gently poured on a double filter paper fitted in a 2 mm mesh sieve, put on a tray with 100 ml tap water and were collected after 48 h of incubation. Potworms were extracted with a Baermann funnel (wet extraction with temperature increases from 20 °C to 45 °C within 3 h) (Moldenke, 1994). Springtails (*F. candida* and remaining *O. cincta*) and mites were extracted using a Berlese funnel (Tullgren funnel, gradual temperature increases from 20 °C to 45 °C in 5 days) (Moldenke, 1994). The extraction efficiency of springtails with the Berlese funnel was rather low. Therefore, we modified our method and extracted springtails by the addition of water to the springtail microcosms and mixing the water and soil by gently stirring. The floating springtails were subsequently removed with a spoon and collected in jars with a layer of gypsum. A (stereo) microscope was used to count the individuals of each of the fauna species.

Experiment II

Experimental set-up. To test the consistency of the results in experiment I across slightly varying soil physical conditions, a second experiment was conducted. The set-up was largely comparable with experiment I, but with a lower hay : soil ratio in the top layer and with fewer faunal treatments. Below we list only the differences between both experiments, all other aspects were the same as in experiment I. Five treatments were included in experiment II: mites, springtails, potworms, all with hay and two control treatments (one with and one without hay addition) (Table 1). The springtail densities of *F. candida* were lower compared with experiment I due to their limited availability; the species *O. cincta* was not included because all individuals had died in experiment I (likely due to the fact that they prefer algae over fungi, J. Ellers, personal communication). Hay residue was applied corresponding to a fertilization rate of 125 kg N ha^{-1} as in experiment I, but was now mixed with 60 g instead of 30 g dry soil rewetted with distilled water (at a dose of 150 ml kg^{-1} dry soil) to form a more compact hay–soil mixture that was put on top of the subsoil layer. Each treatment had five replicates in which fluxes of N_2O and CO_2 were measured during 59 days, and soil analyses and fauna extractions were conducted on day 62. No destructive samples were taken during the course of the experiment.

Experiment III

Experimental set-up. To test the effect of faunal density on N_2O emissions (hypothesis 3) a third, 56-day experiment was set-up. Experimental details (including the hay : soil ratio in the top layer) were identical to experiment II, apart from the soil fauna treatments which comprised the inoculation of potworms at a density of 0, 10, 50 or 100 potworms per microcosm (Table 1). We chose potworms to test density effects given their significant and opposing impact on N_2O emissions in experiments I and II. The potworms were introduced after 3 days preincubation of the microcosms. Five replicates were used for flux measurements, and an additional three were used for destructive soil analyses and faunal extractions on day 35.

Data processing and statistical analysis

Cumulative N_2O and CO_2 emissions were calculated assuming a linear change in emission rates between the subsequent flux measurements (Lubbers *et al.*, 2011). Treatment effects were tested by one-way analysis of variance (ANOVA) and differences between treatment levels by post hoc Tukey tests using the statistical program GenStat (14th edition software package). The control soil treatment without hay was not included in any statistical analysis of experiments I, II and III, and solely served as a baseline reference in the figures. Instead, soil with hay residue but without fauna served as control treatment in all three experiments.

For experiments I and II, the effect of fauna treatments on soil parameters as well as on N_2O and CO_2 emissions was analysed by multivariate redundancy analysis (RDA) and Monte Carlo permutation tests (999 unrestricted permutations; CANOCO for Windows version 4.5, Plant Research International, Wageningen, The Netherlands). RDA diagrams were made for the dates of destructive sampling: days 34 and 62 for experiment I and day 62 for experiment II. For experiment I, measured N_2O and CO_2 fluxes on days 32 and 35 were used to estimate fluxes on day 34 by linear interpolation. These fluxes were subsequently linked with soil parameter data from microcosms in the same block with the same treatment.

In experiment III, we used repeated measures ANOVA on actual N_2O emission rates of 18 sampling points in time to test

for differences between potworm density treatments. Treatment differences in cumulative N_2O emissions at days 35 and 56 were tested using one-way ANOVA, followed by post hoc Tukey tests.

Results

Experiment I

N_2O and CO_2 emissions. Soil fauna treatments significantly affected N_2O fluxes from soil in which hay (low C : N ratio) was incorporated into experiment I (Fig. 1a). Soil fauna also had a significant effect ($P < 0.001$) on the cumulative N_2O emissions after 61 days of incubation (Fig. 1b). Earthworms and potworms significantly decreased cumulative N_2O emissions compared with the control hay treatment from 78.4 in the control to 37.0 in the earthworm and 53.5 mg N_2O-N m^{-2} in the potworm treatment. Highest cumulative N_2O fluxes were measured in the springtail treatment (90.2 mg N_2O-N m^{-2}), but these did not differ significantly from the control with hay. After 20 days of incubation, N_2O emissions gradually started to increase until maximum fluxes were measured on day 37, except for the earthworm treatment which showed an early peak of N_2O emissions and the springtail treatment which delayed the peak of N_2O emissions (Fig. 1a).

Cumulative CO_2 emissions after 61 days differed between treatments ($P < 0.001$), but no single treatment differed significantly from the control hay treatment (Fig. 1c). Highest cumulative CO_2 emissions were measured for the potworm (54.3 g CO_2-C m^{-2}) and lowest for the nematode treatment (47.8 g CO_2-C m^{-2}). CO_2 emissions were positively correlated with total N and NO_3 in both experiments I and II (Figs 2 and 4) and indicate a release of mineral N during decomposition. However, the factors explaining subsequent N_2O emissions were not consistent across experiments I and II, as shown by the RDA diagrams. Whereas other studies reported positive correlations between NO_3 and N_2O emissions (Firestone *et al.*, 1980; Davidson *et al.*, 2000), we did not find these correlations in experiment I (Fig. 2a and b). Therefore, we hypothesize that another factor than decomposition rate and N availability was the main controller of N_2O emission in experiment I, possibly soil aeration. However, in experiment II, the availability of N and C was probably the main controlling factors of N_2O emissions, given the positive correlations between CO_2 emissions, NO_3 and N_2O emissions (Fig. 4).

Faunal abundance and soil parameters. Faunal numbers showed clear dynamics over time (Table 3). Nematode and mite abundance declined during the experiment. Potworms and *F. candida* springtails reproduced, whereas the abundance of the springtail *O. cincta* gradually declined until no individuals were alive shortly after the second destructive sampling (visual observation of the microcosms). Earthworms did not reproduce, but

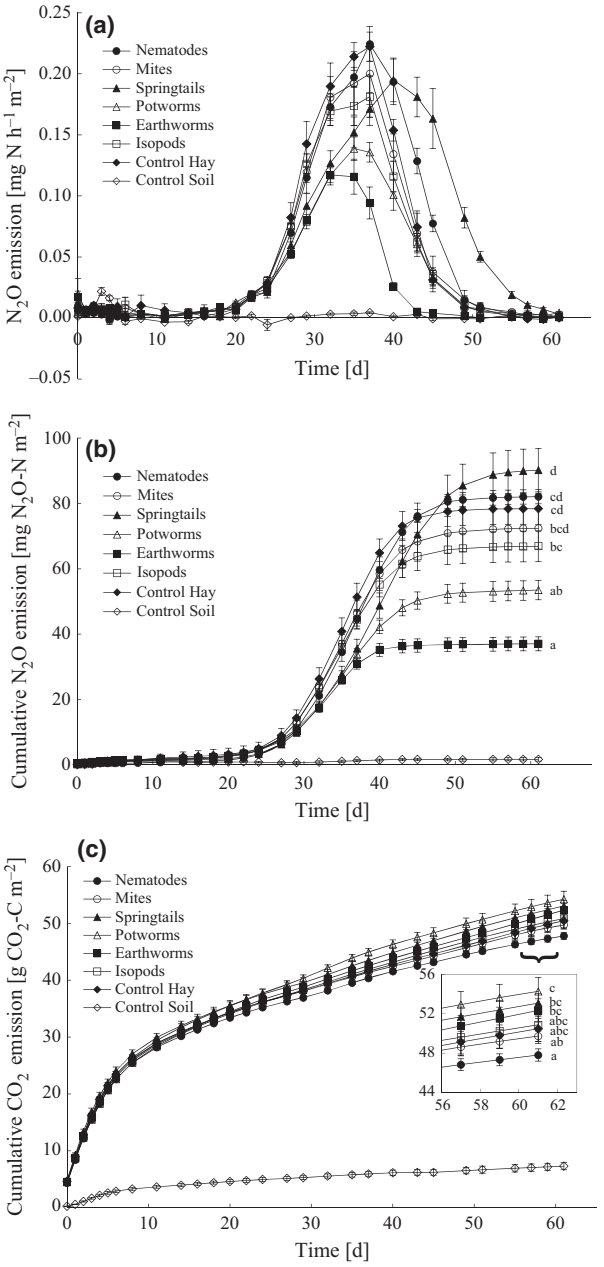

Fig. 1 N_2O and CO_2 emissions in experiment I with actual N_2O emissions (a), cumulative N_2O emissions (b) and cumulative CO_2 emissions (c) in time (d = days). Error bars indicate SEs ($n = 5$). Letters denote significant differences between treatments of cumulated N_2O and CO_2 emissions on day 61 ($P < 0.001$). The control soil treatment was not included in the ANOVA.

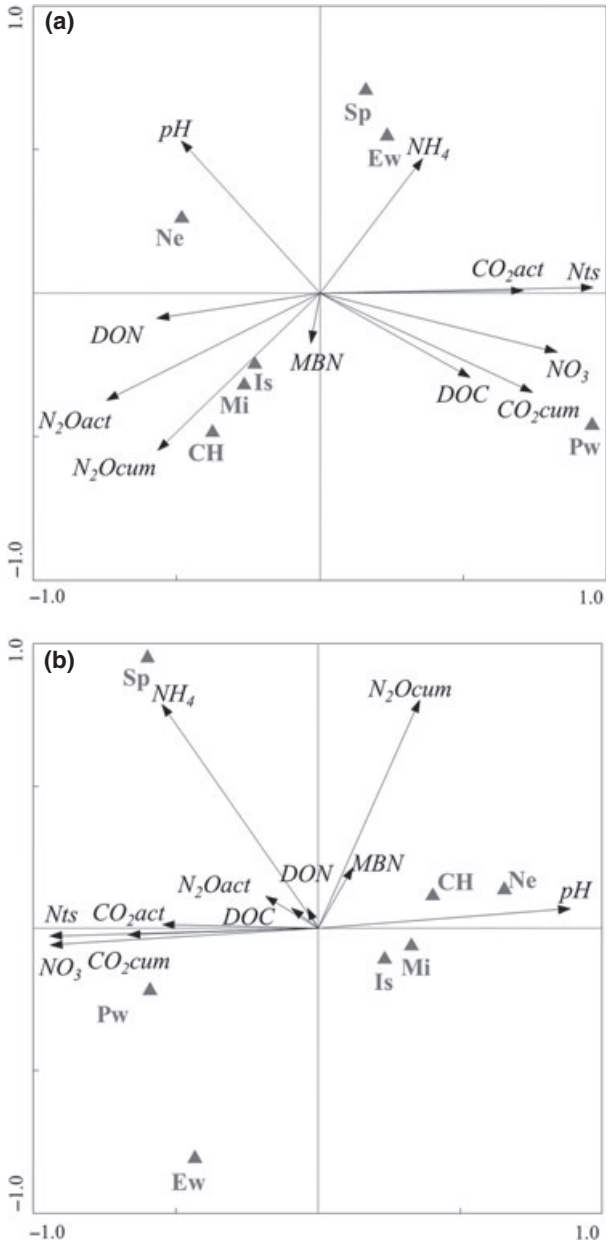

Fig. 2 Redundancy analysis (RDA) diagrams of soil parameters and N_2O and CO_2 emissions in relation to fauna treatments for experiment I on day 34 (a) and day 62 (b). Codes refer to treatments listed in Table 1, act = actual emissions, cum = cumulative emissions, DOC = dissolved organic carbon, DON = dissolved organic nitrogen, MBN = microbial biomass nitrogen, Nts = total nitrogen.

their survival was high as only one individual was found dead after 34 days. Two isopods did not survive, and in three of five replicates, some isopod juveniles were found at the end of the experiment. Four of five isopod replicates at final harvest were contaminated with mites, with an average 13×10^3 mites m^{-2}. The mite species was not identified, but was neither one of the species used in the mite treatment and was probably inoculated from the isopod culture.

Control hay and control soil treatments were analysed for potential contamination with nematodes, mites, springtails or potworms at the end of the experiment. Bacterial-feeding nematodes were found (272 (±34) × 10^4 nematodes m^{-2} in the control hay treatment and 114 (±36) × 10^3 nematodes m^{-2} in the control soil treatment, $n = 2$). These nematodes were classified in the family of *Rhabditidae*, genus *Mesorhabditis*. Their occurrence in the control soil treatment indicates that they survived the defaunation of the soil. Therefore, these bacterial-feeding nematodes were likely present in the other treatments as well.

Soil mineral N, MBN, DOC and pH data are presented in Table 4. During the experiment, NH_4, DON, DOC and pH levels decreased, whereas NO_3 increased. Fauna treatments showed significant effects on some of these soil parameters, but their effects changed over time. MBN was highest at day 13 and decreased till low levels at day 62, when the significant effect of soil fauna on MBN had disappeared.

Relation between fluxes, fauna and soil parameters. Multivariate redundancy analysis at day 34 showed that the soil fauna treatments explained 64.7% ($P = 0.001$) of the total variation in our measured soil parameters and N_2O and CO_2 emissions across all fauna treatments (Fig. 2a). The first canonical axis, mainly determined by actual CO_2 emissions, total N and DON, explained 40.8% of the total variation. The second axis was determined by NH_4 and pH and explained an additional 11.1% of the variation. The N_2O emissions correlated strongly positively with the control hay treatment and strongly negatively with the earthworm and springtail treatments. The potworm treatment was strongly positively related to CO_2 emissions, NO_3 and total N in soil, but did not correlate with N_2O emissions.

At day 62, the total variation in the emissions and soil parameters was explained for 56.4% ($P = 0.001$) by the soil fauna treatments. The first canonical axis was mainly determined by NO_3, total N, CO_2 emissions and pH, and explained 34.5% of the total variation. The second canonical axis included cumulative N_2O emission and NH_4, and explained 12.2% of the variation (Fig. 2b). The potworm treatment was still strongly positively correlated with CO_2 emissions as well as NO_3 and total N content, whereas a negative correlation with cumulative N_2O emissions was found. The earthworm treatment still correlated strongly negatively with cumulative N_2O emissions, whereas the effect of springtails on these emissions switched from negative (after 34 days) to positive.

Table 3 Fauna numbers in the six fauna treatments of experiment I, counted after 13, 34 and 62 days, expressed in individuals m^{-2} and their relative increase/decrease compared with added number of individuals on day 0

	13 days		34 days		62 days	
	(# m^{-2})	(%)	(# m^{-2})	(%)	(# m^{-2})	(%)
Ne	161 (±53) × 10^3	−48	752 (±14) × 10^2	−76	137 (±97) × 10^3	−56
Mi	160 (±40) × 10^3	33	325 (±87) × 10^3	171	715 (±38) × 10^2	−40
Sp: *Folsomia candida*	178 (±34) × 10^2	−52	464 (±180) × 10^3	1154	130 (±21) × 10^4	3409
Orchesella cincta	314 (±19) × 10^2	−15	511 (±57) × 10^1	−86	0 (±0)	−100
Pw	555 (±10) × 10^1	−63	110 (±20) × 10^3	632	455 (±18) × 10^3	2933
Ew	570 (±0)	0	473 (±95)	−17	570 (±0)	0
Is	140 (±0) × 10^1	0	140 (±0) × 10^1	0	330 (±0) × 10^1	135

Values are indicated with SE between brackets (n = 3 on days 13 and 34, n = 5 on day 62). Codes refer to treatments listed in Table 1.

Experiment II

N_2O and CO_2 emissions. The potworm treatment resulted in highest cumulative N_2O emissions after 59 days (123.5 mg N_2O-N m^{-2}) and was significantly higher ($P < 0.001$) than the mite, control hay and springtail treatments (62.5, 51.9 and 50.1 mg N_2O-N m^{-2}, respectively, Fig. 3a).

Cumulative CO_2 emissions showed similar results, with significantly higher emission ($P < 0.001$) of the potworm treatment (40.5 g CO_2-C m^{-2}), compared with the control hay treatment (35.4 g CO_2-C m^{-2}, Fig. 3b).

Faunal abundance and soil parameters. Springtail and potworm abundances increased, whereas mites decreased in abundance (Table 5). Contamination of bacterial-feeding nematodes was tested by extractions from control hay and control soil treatments (n = 2), and resulted in 243 (±119) × 10^4 and 79 (±44) × 10^3 individuals m^{-2} respectively.

Soil NH_4, (NO_3 + NO_2) and DON were affected by fauna treatments (Table 6). Potworm treatments always resulted in significantly higher soil N levels compared with the other treatments. Fauna treatments also affected MBN and pH, with highest and lowest values, respectively, for the potworm treatment. DOC was not significantly affected by fauna treatments.

Relation between fluxes, fauna and soil parameters. The RDA diagram of experiment II and tests of the significance of the ordination axes show that the fauna treatments explained a total variation of 57.1% (P = 0.001) of the soil and flux parameters (Fig. 4). Many factors contributed to the first canonical axis which explained 45.4% of the total variation: total N, NO_3, cumulative N_2O and CO_2 emissions, DON and NH_4. Actual CO_2 emissions and MBN contributed to the second canonical axis, but this axis only explained 8.2% of the total variation.

Experiment III

N_2O and CO_2 emissions. The density of potworms significantly affected the N_2O emissions (repeated measure ANOVA within treatments, $P < 0.001$; and interaction between time and treatments, $P < 0.001$). The peak in actual N_2O emission rate appeared earlier in the treatments with high densities compared with treatments with moderate or low densities of potworms (Fig. 5). After 35 days, the cumulative N_2O emission was significantly different between the treatments ($P < 0.001$). From microcosms with high densities of potworms the N_2O emission on day 35 was nearly four times higher than that of microcosms with low densities of potworms. The cumulative N_2O emissions after 56 days, however, were similar for all potworm density treatments (Fig. 6).

Faunal abundance and soil parameters. The control treatments remained free of potworms, whereas in all other treatments the potworms established well and their abundance doubled to tripled by the end of the experiment (results not shown).

Soil mineral N on day 35 was significantly affected by potworm density (Table 7), with highest concentrations in the highest potworm density treatment. At the end of the experiment, nitrate levels still increased with potworm density ($P < 0.001$). DOC concentrations also increased significantly with increasing potworm density at day 35 (P = 0.040). At day 56 the treatment effects were still significant (P = 0.014), but highest concentrations were not found at the higher potworm densities but rather in the control and lower potworm densities (Table 7).

Table 4 Soil parameters over time in experiment I

	NH_4 (mg N kg^{-1} soil)			$NO_3 + NO_2$ (mg N kg^{-1} soil)		
	Day 13	Day 34	Day 62	Day 13	Day 34	Day 62
Ne	11.9 (±0.26)abc	5.3 (±2.4)	0.06 (±0.02)a	4.19 (±0.30)	20.8 (±2.5)a	40.2 (±0.6)a
Mi	13.5 (±0.09)c	2.1 (±0.8)	0.06 (±0.01)a	4.55 (±0.17)	30.5 (±2.3)bc	45.6 (±1.8)a
Sp	12.6 (±0.46)bc	8.5 (±2.3)	1.02 (±0.11)c	4.22 (±0.13)	27.2 (±2.4)ab	62.8 (±0.6)b
Pw	16.3 (±0.07)d	6.4 (±2.7)	0.29 (±0.04)b	4.63 (±0.17)	40.1 (±1.0)c	63.0 (±2.4)b
Ew	13.1 (±0.63)bc	7.1 (±0.6)	0.00 (±0.02)a	5.09 (±0.16)	32.2 (±0.6)bc	62.3 (±1.2)b
Is	9.8 (±0.23)a	3.1 (±1.2)	0.10 (±0.02)ab	4.36 (±0.12)	27.0 (±1.6)ab	45.1 (±1.9)a
CH	10.7 (±1.05)ab	4.2 (±1.0)	0.10 (±0.03)ab	4.22 (±0.13)	23.8 (±1.4)ab	41.8 (±1.6)a
CS	8.8 (±0.25)	2.2 (±1.2)	0.00 (±0.01)	5.56 (±0.02)	15.2 (±0.7)	21.5 (±0.8)
ANOVA	<0.001	ns	<0.001	ns	<0.001	<0.001
	DON (mg N kg^{-1} soil)			DOC (mg C kg^{-1} soil)		
	Day 13	Day 34	Day 62	Day 13	Day 34	Day 62
Ne	10.6 (±0.1)abc	9.9 (±0.5)	3.1 (±0.3)	22.0 (±1.0)	17.4 (±0.3)	15.1 (±0.4)
Mi	12.2 (±0.5)c	8.5 (±0.5)	3.6 (±0.4)	26.0 (±2.2)	16.6 (±1.1)	14.2 (±0.7)
Sp	12.0 (±0.5)bc	8.9 (±0.7)	3.6 (±0.2)	23.6 (±1.2)	17.6 (±0.9)	14.7 (±1.0)
Pw	10.9 (±0.5)abc	7.9 (±0.0)	2.8 (±0.3)	26.5 (±3.9)	20.7 (±1.4)	16.0 (±0.7)
Ew	10.2 (±0.3)ab	7.8 (±0.2)	3.5 (±0.5)	20.4 (±0.4)	17.9 (±0.6)	14.3 (±0.3)
Is	9.9 (±0.4)a	9.2 (±0.9)	3.1 (±0.2)	21.6 (±0.7)	18.0 (±0.5)	13.8 (±0.3)
CH	11.3 (±0.3)abc	10.1 (±0.7)	3.2 (±0.2)	22.3 (±1.3)	18.6 (±0.8)	15.3 (±0.3)
CS	3.7 (±0.1)	3.7 (±0.5)	2.5 (±0.1)	7.2 (±0.1)	7.2 (±0.8)	6.2 (±0.2)
ANOVA	<0.01	ns	ns	ns	ns	ns
	MBN (mg N kg^{-1} soil)			pH		
	Day 13	Day 34	Day 62	Day 13	Day 34	Day 62
Ne	47.0 (±5.9)ab	29.2 (±3.2)b	9.9 (2.2)	6.48 (±0.01)	6.39 (±0.04)	6.05 (±0.01)c
Mi	53.8 (±4.3)b	19.7 (±2.4)a	3.7 (±2.7)	6.55 (±0.02)	6.29 (±0.06)	6.00 (±0.02)c
Sp	42.9 (±4.3)ab	16.3 (±1.5)a	6.5 (±3.2)	6.53 (±0.01)	6.36 (±0.04)	5.85 (±0.03)b
Pw	50.5 (±2.5)ab	25.1 (±2.0)ab	7.2 (±3.3)	6.54 (±0.03)	6.18 (±0.06)	5.77 (±0.02)a
Ew	34.7 (±1.7)ab	20.3 (±1.8)ab	1.6 (±3.7)	6.55 (±0.01)	6.35 (±0.02)	5.88 (±0.01)b
Is	33.4 (±1.5)a	16.9 (±2.7)a	6.2 (±2.0)	6.54 (±0.01)	6.31 (±0.04)	5.98 (±0.02)c
CH	40.2 (±5.2)ab	23.1 (±1.5)ab	5.5 (±1.4)	6.54 (±0.01)	6.30 (±0.03)	6.05 (±0.02)c
CS	2.6 (±0.0)	0.0 (±1.1)	0.0 (±0.6)	6.21 (±0.02)	6.00 (±0.01)	5.86 (±0.01)
ANOVA	<0.05	<0.01	ns	ns	ns	<0.001

Values are indicated with SE between brackets (n = 3 on days 13 and 34, n = 5 on day 62).
Letters denote significant differences; control soil treatment (CS) was not included in the ANOVA. ns, not significant, DON = dissolved organic nitrogen, DOC = dissolved organic carbon, MBN = microbial biomass nitrogen. Codes refer to treatments listed in Table 1.

Discussion

Our results show that soil invertebrate fauna can significantly increase, decrease, accelerate or delay N_2O emissions. The actual influence that fauna exert on N_2O emissions, however, depends on their functional group as well as on the soil physical and chemical soil parameters. These relations will be explored further below.

Faunal effects on N_2O emissions

Overall, experiments I and II showed that soil invertebrate fauna can affect N_2O emission, and that different functional faunal groups affect N_2O emissions differently (Figs 1a, b and 3a), confirming hypotheses 1 and 2. Our results are in line with studies on faunal contributions to N mineralization, which reported that after amoeba and bacterial-feeding nematodes, earthworms and potworms are the most important faunal groups contributing to N mineralization (Osler & Sommerkorn, 2007; Moore & de Ruiter, 2012). This sequence of importance is reflected in our experiment, where earthworms and potworms showed largest effects on N_2O emissions and on soil mineral N availability (Table 4). Effects of fungivorous nematodes, mites, springtails and isopods on N_2O emissions were not significant,

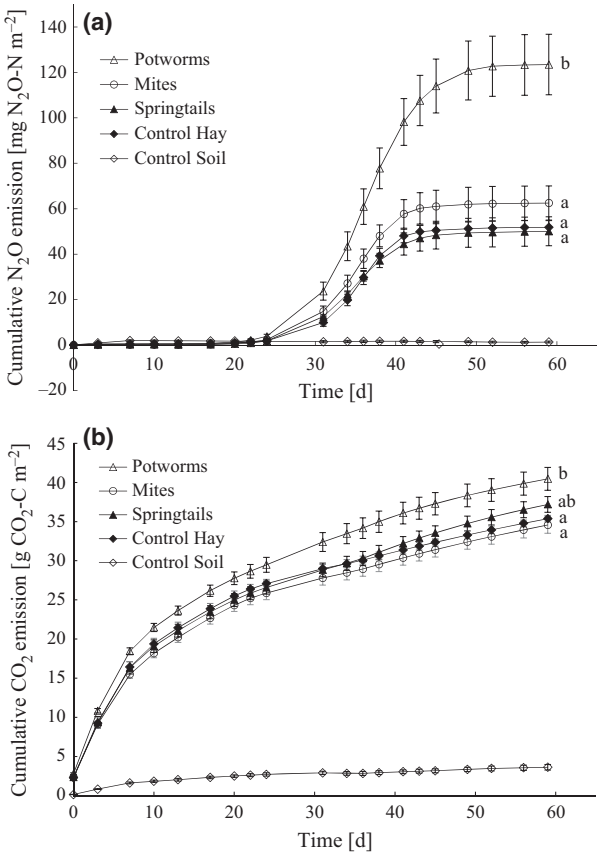

Fig. 3 Cumulative emissions of N_2O (a) and CO_2 (b) in experiment II. Error bars indicate SEs (n = 5). Letters denote significant differences between treatments of cumulated emissions on day 59 ($P < 0.001$). The control soil treatment was not included in the ANOVA.

Table 5 Fauna numbers in the three fauna treatments with hay of experiment II, counted after 62 days, expressed in individuals m^{-2} and their relative increase/decrease compared with added number of individuals on day 0

	(# m^{-2})	(%)
Mi	172 (±37) × 10^2	−86
Sp	104 (±28) × 10^4	3357
Pw	393 (±20) × 10^3	2522

Values are indicated with SE between brackets (n = 5). Codes refer to treatments listed in Table 1.

corresponding to negligible contributions of microarthropods to N mineralization found by de Ruiter (1993). This was mainly attributed to their relatively low biomass (Filser, 2002). The presence of bacterial-feeding nematodes (and probably protozoa) in all treatments (including the controls) might have led to an underestimation of total faunal impacts on N_2O emissions, as they are known to exert the most significant faunal contributors to N mineralization (de Ruiter *et al.*, 1995; Osler & Sommerkorn, 2007) and therefore probably to higher N_2O emissions as well.

Across both experiments I and II, only potworms and earthworms showed significant suppressing or enhancing effects on cumulative N_2O emissions. As these are the only fauna species in our experiments that were able to disrupt the soil structure by their feeding and burrowing activities (Verhoef & Brussaard, 1990), the observed N_2O effects may be related to their ability to modify the soil physical structure.

The observed reduction in cumulative N_2O emissions in the presence of earthworms and potworms in experiment I was contrary to our first hypothesis. For earthworms, we speculate that this might be an effect of the redistribution of organic matter into the lower soil layer, which was only (visually) observed in the earthworm treatment. Produced N_2O in this lower soil layer had a longer diffusion pathway than N_2O produced in the upper hay–soil mixture, which may have resulted in more N_2O reduction and consequently lower net N_2O emissions (Chapuis-Lardy *et al.*, 2007; Paul *et al.*, 2012).

Opposing potworm effects

Surprisingly, potworms decreased N_2O emissions in experiment I, but increased emissions in experiment II, while the experimental set-ups only slightly differed. Similar to earthworms, potworms are known to increase mobilization of N and to affect nitrification by microbial grazing and their burrowing activities (van Vliet *et al.*, 2004). Casts of potworms are rich in NH_4 (Maraldo *et al.*, 2011) and might promote denitrification, as earthworms do (Drake & Horn, 2006). This may have led to enhanced N_2O production.

In experiment I, this stimulating effect of potworms on N_2O production was probably outweighed by their effect on the soil structure. Such an effect was also discussed by van Vliet *et al.* (2004), who reported reduced N_2O emissions in a loamy sandy soil with surface litter treatment in the presence of potworms. The hay–soil mixture in experiment I had initially a very low bulk density, and potworms might have caused a compaction of the hay–soil mixture due to their burrowing activities (this was also visually observed). As a result, more complete reduction of NO_3 to N_2 may have occurred, leading to less N_2O emission.

In contrast, potworms increased emissions in experiment II, which had a higher initial density of the hay–soil mixture than experiment I, and therefore probably more anaerobic conditions and higher denitrification rates. These conditions may have resulted

Table 6 Soil parameters for experiment II on day 62

	NH_4 (mg N kg^{-1} soil)	$NO_3 + NO_2$ (mg N kg^{-1} soil)	DON (mg N kg^{-1} soil)	DOC (mg C kg^{-1} soil)	MBN (mg N kg^{-1} soil)	pH
Mi	0.23 (±0.04)a	34.6 (±0.5)a	9.0 (±0.5)a	11.4 (±0.7)	4.7 (±0.9)a	6.08 (±0.02)bc
Sp	1.08 (±0.14)b	41.2 (±1.3)b	9.3 (±0.3)a	11.1 (±0.6)	5.6 (±0.6)a	6.00 (±0.03)b
Pw	1.12 (±0.05)b	51.9 (±1.5)c	11.1 (±0.5)b	12.9 (±0.7)	8.9 (±2.1)ab	5.88 (±0.02)a
CH	0.27 (±0.02)a	32.3 (±0.8)a	8.9 (±0.5)a	11.8 (±0.7)	14.1 (±1.8)b	6.13 (±0.03)c
CS	0.13 (±0.06)	13.6 (±0.6)	3.1 (±0.1)	3.8 (±0.1)	0.0 (±0.8)	5.74 (±0.02)
ANOVA	<0.001	<0.001	0.005	ns	0.005	<0.001

Values are indicated with SE between brackets (n = 5).
Letters denote significant differences; control soil (CS) treatment was not included in the ANOVA. ns = not significant, DON = dissolved organic nitrogen, DOC = dissolved organic carbon, MBN = microbial biomass nitrogen. Codes refer to treatments listed in Table 1.

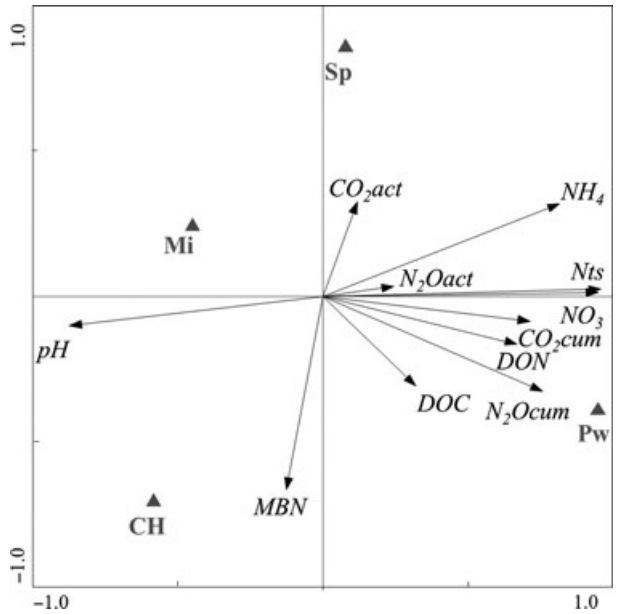

Fig. 4 Redundancy analysis (RDA) diagram of soil parameters and N_2O and CO_2 emissions in relation to fauna treatments with hay for experiment II at day 62. Codes refer to treatments listed in Table 1, act = actual emissions, cum = cumulative emissions, DOC = dissolved organic carbon, DON = dissolved organic nitrogen, MBN = microbial biomass nitrogen, Nts = total nitrogen.

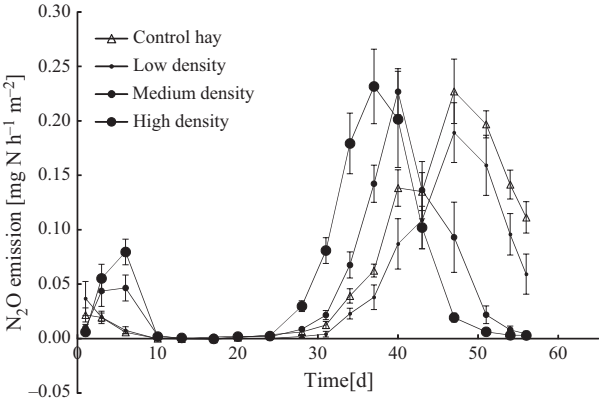

Fig. 5 Actual N_2O emissions over time, for experiment III with different densities of potworms. Low, medium and high density of potworms denotes 10, 50 and 100 individuals per microcosm respectively (see Table 1). Error bars indicate SEs (n = 5).

in lower cumulative CO_2 emissions, and lower NO_3 values in experiment II (Table 6) compared with experiment I. We hypothesize that, under those conditions, potworm effects on N mineralization and C availability were relatively more important than their effect on the soil structure. This is corroborated by the correlations between NH_4, NO_3 and CO_2 and N_2O emissions, all pointing towards the potworm treatment (Fig. 4).

Fauna density effects

Higher densities of potworms in experiment III accelerated rather than increased total N_2O emissions, thereby partly confirming our third hypothesis (Figs 5 and 6). This is in line with earlier studies that found that the N mineralization rate was stimulated by higher potworm densities (Nieminen 2010). Over time, however, food source depletion is likely to occur earlier with higher density of soil fauna (Gómez-Brandón *et al.*, 2010) such that increased N mineralization is not maintained over time. We indeed found that mineral N availability at midterm of the experiment increased with potworm density. These differences were, however, also still notable at the end of the experiment, indicating that N availability alone cannot explain the observed differences in timing of N_2O emissions. One of the important parameters may well be carbon availability for the heterotrophic denitrifiers. We indeed found higher concentrations of DOC with increased densities of potworms at midterm harvest, but at much reduced levels, especially at high and moderate density of potworms, at the end of the experiment (Table 7).

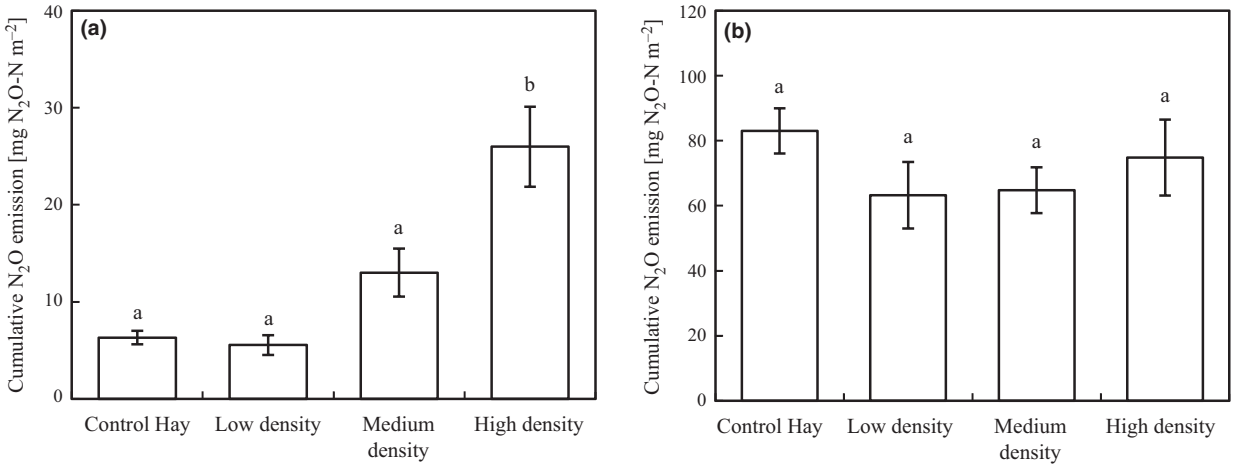

Fig. 6 Cumulative N_2O emissions for experiment III on day 35 (a) and day 56 (b). Low, medium and high density of potworms denotes initial densities of 10, 50 and 100 individuals per microcosm respectively. Error bars indicate SEs, different letters indicate significant differences between treatments ($P < 0.05$).

Table 7 Soil parameters for experiment III on days 35 and 56

	Day 35			Day 56		
	NH_4 (mg N kg^{-1} soil)	$NO_3 + NO_2$ (mg N kg^{-1} soil)	DOC (mg C kg^{-1} soil)	NH_4 (mg N kg^{-1} soil)	$NO_3 + NO_2$ (mg N kg^{-1} soil)	DOC (mg C kg^{-1} soil)
Low	20.9 (±1.1)a	4.8 (±0.6)a	117.3 (±17.1)ab	8.6 (±0.6)	26.2 (±1.2)ab	86.0 (±4.4)b
Medium	23.6 (±1.4)ab	6.7 (±0.8)ab	105.3 (±2.7)ab	7.2 (±0.6)	32.2 (±2.3)bc	67.0 (±3.3)a
High	28.4 (±0.9)b	9.8 (±0.4)b	137.0 (±5.2)b	6.8 (±0.4)	37.8 (±1.6)c	69.0 (±2.4)ab
CH	19.8 (±1.3)a	5.6 (±0.9)a	89.3 (±9.8)a	8.0 (±0.5)	20.6 (±1.5)a	84.4 (±7.0)ab
ANOVA	0.012	0.008	0.040	ns	<0.001	0.014

Values are indicated with SE between brackets ($n = 5$)
Letters denote significant differences. Codes refer to treatments listed in Table 1. Low, medium and high density of potworms denotes initial densities of 10, 50 and 100 individuals per microcosm respectively.

Future perspectives

This is the first study showing that different invertebrate soil fauna species can significantly affect N_2O emissions from soil. Ecosystem engineers (earthworms and potworms) showed largest effects on N_2O emissions, most likely due to their ability to change the soil structure. Ultimately, faunal effects on N_2O emissions depend on the complex interplay between their effects on the availability of N, C and gas diffusion (especially of O_2 into the soil and N_2O out of the soil).

Further research should focus on different parameters of such food webs. For example, the effects of faunal densities of different groups of soil fauna, interactions between functional faunal groups, effects between trophic levels and effects of residue quality, as all these factors are known to influence N mineralization in soil (Cragg & Bardgett, 2001; Cole *et al.*, 2004; Kaneda & Kaneko, 2008). Therefore, these soil fauna community attributes are likely to affect N_2O emissions as well. As relations between faunal diversity and N_2O emissions are likely to be complex, we suggest that a systematic study of N_2O fluxes from artificial food webs of increasing complexity within and between trophic levels would be the first step for such an approach.

A better understanding of the role of soil fauna on N_2O emissions will contribute to further improvements of N_2O modelling. This knowledge, in relation to well-understood effects of agricultural management practices on soil food webs (Roger-Estrade *et al.*, 2010), can form a basis to develop future N_2O mitigation strategies.

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