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SHORT COMMUNICATION

Manure-associated stimulation of soil-borne methanogenic activity in agricultural soils

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Abstract The growing human population and scarcity of arable land necessitate agriculture intensification to meet the global food demand. Intensification of agricultural land entails manure input into agrosystems which have been associated to increased methane emission. We investigated the immediate short-term response of methane production and the methanogens after manure amendments in agricultural soils and determined the relevance of the manure-derived methanogenic population in its contribution to soil methane production. We followed methane production in a series of unamended and manure-amended batch incubations: (i) manure and soil, (ii) sterilized manure and soil, and (iii) manure and sterilized soil. Moreover, we determined the methanogenic abundance using a quantitative PCR targeting the *mcrA* gene. Results show that the soil-borne methanogenic community was significantly stimulated by manure amendment, resulting in increased methane production and *mcrA* gene abundance; manure-derived methanogenic activity contributed only marginally to overall methane production. Accordingly, our results highlighted the importance of the resident methanogenic community and physiochemical properties of a residue when considering methane mitigation strategies in agricultural soils.

Keywords Methane production · Methanogens · Manure-amendment · *mcrA* · Agricultural management

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Introduction

With the growing human population and scarcity of arable land, agriculture is intensified to meet the global food demand. Agriculture-derived methane including ruminants accounts for approximately 27 % of the total global methane budget of 500–600 Tg methane annually (Conrad 2009). Although methane evolution to atmosphere has slowed and remained relatively constant from the 1990s till 2006, from then onwards, there has been a steady increase (Nisbet et al. 2014) and is a cause for global concern. The effects of agriculture, including manure input in fields with regard to greenhouse gas emissions have been widely studied (Ma et al. 2012; Skinner et al. 2014). Indeed, the input of animal excrements (manure) in agrosystems, a typical agricultural practice, has been associated with increased methane emission (Radl et al. 2007; Thangarajan et al. 2013). It was suggested that with the addition of manure, methanogens indigenous to the rumen are inoculated into the soil, potentially contributing to higher methane emission (Radl et al. 2007; Gattinger et al. 2007; Kim et al. 2014a). Alternatively, the addition of organic matter into the soil may stimulate soil-borne methanogens (Gattinger et al. 2007). These studies documented the microbial communities, including the methanogens in manure-amended soils (e.g., cattle-impacted field) and soils without manure amendment (reference site). The methanogenic community was clearly different in soils after long-term manure amendment, suggesting that regular manure application selected for specific methanogens (Gattinger et al. 2007). While community analysis of the methanogens showed an effect of manure input in agricultural soils, shifting the microbial composition to a predominance of specific strains, it is yet unclear whether manure-derived methanogens contributed to the total methane production. The determination of the relative contribution of manure-derived and soil-borne methanogenic community to total methane production is further confounded by the

overlaying methanogenic community composition in the soil and manure.

To date, it remains unresolved whether manure amendment increased methane production in soil is due to (i) stimulating soil-borne methanogenic community and/or (ii) increasing the soil methanogenic community size with methanogens seeded from the manure. In contrast to previous studies, we explicitly determined and resolved the immediate short-term relevance of the manure-associated methanogenic community in its contribution to soil methane production in laboratory-scale batch incubations.

Materials and methods

Soil and manure description

Focusing on agricultural soils, we used air-dried soils from a rice paddy (CRA Agriculture Research Council, Rice Research Unit, Vercelli, Italy; coordinates 45° 20′ N, 8° 25′ W) and potato cultivated field (Applied Plant Research Unit, Wageningen University, Lelystad, The Netherlands; coordinates 52° 31′2 0″ N, 05° 34′ 57″ E) representing a wetland and well-aerated (i.e., oxic) agricultural system, respectively. Manure from the center of four fresh cow dung pads was sampled and mixed (composite sample), immediately wet sieved (2 mm), and stored in the 4 °C fridge till incubation setup. An aliquot of the sieved manure was used to determine the physiochemical parameters. Selected physiochemical soil and manure characteristics are given in Table 1.

Incubation setup and methane measurement

To achieve a standardize incubation condition, soil, autoclaved deionized water, and manure were homogenized by vortex in a beaker and subsequently distributed into four bottles (four replicate per treatment). Each bottle comprised 5 g soil and 5 ml autoclaved deionized water with (treatment incubation) and without (reference incubation) manure amendment. The soils were amended with manure at 10, 20, and 40 % dry weight soil. In addition, incubations containing thrice-autoclaved (sterilized) manure and gamma-irradiated (sterilized) soils served as controls. The bottle was capped with a butyl rubber stopper and flushed with N_2 for 30 min before the initiation of incubation. Incubations were performed at 25 °C in the dark while shaking (120 rpm). The incubation setup was performed aseptically.

To differentiate the contribution of methane production from soil-borne and manure-seeded methanogens, incubations considering combinations of manure, soil, sterilized manure, and sterilized soil were set up

Table 1 Selected soil and manure properties

Soil/manure	Geographic origin	Soil texture	pH (KCl)	Organic matter content (%)[a]	Total carbon (μg C mg dw sample^{-1})	Total nitrogen (μg N mg dw sample^{-1})	C/N	Soil nutrient contents[b] (μg g dw sample^{-1})			Vegetation cover[c]
								NO_x	NH_4^+	PO_4^{3-}	
Wetland soil	Vercelli (Italy)	Clay	5.41±0.02	3.98±0.11	13.90±0.60	1.29±0.05	19.74±0.23	34.38±0.93	18.03±0.43	0.59±0.42	Fallow (rice paddy)
Oxic soil	Lelystad (The Netherlands)	Clay	7.55±0.02	5.08±0.18	27.26±1.67	1.60±0.06	17.25±0.48	9.06±0.46	7.85±0.24	0.79±0.02	Fallow (potato)
Manure	Wageningen (The Netherlands)	n.a.	7.92±0.05	81.08±2.56	383.33±7.80	18.73±0.61	20.48±0.70	136.77±47.74	6272.73±139.46	10,203.04±123.27	n.a

Measurements were performed in triplicates (mean±s.d.)

[a] Loss of ignition (%)

[b] During sampling (crop)

[c] Total content (soluble and adsorbed fractions) determined in KCl (1:5). NO_x refers to the total of NO_2^- and NO_3^-

independently for both soils, giving a total of 104 incubations. The manure-amended incubations contained (i) manure and soil, (ii) sterilized manure and soil, and (iii) manure and sterilized soil at different manure input (Fig. 1). During incubation (10 days), methane in the headspace was measured using an Ultra GC gas chromatograph (Interscience, The Netherlands) equipped with a flame ionization detector (FID) and at Rt-Q-Bond (30 m, 0.32 mm, ID) capillary column. We used helium as a carrier gas and set oven temperature at 80 °C. After incubation, soil was homogenized by vortex, sampled, and kept in aliquots in the −20 °C freezer till DNA extraction.

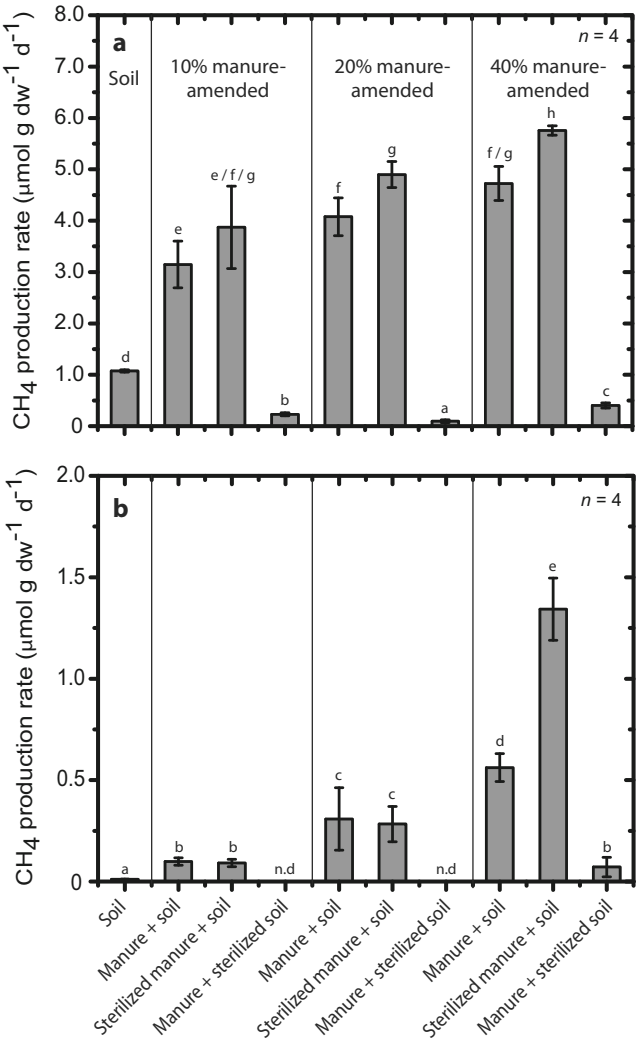

Fig. 1 Methane production rate in manure-amended incubations (10, 20, and 40 $\%_{w/w}$ amendments) and incubation without manure addition for wetland (**a**) and oxic (**b**) agricultural soils (mean±s.d.; n=4). The manure-amended incubations include (i) manure and soil, (ii) sterilized manure and soil, and (iii) manure and sterilized soil. The different *letters* indicate statistical significance (ANOVA; $p<0.05$) between treatments. Note the different scales on the *y*-axis. *n.d.* not detected

Nutrient analysis

Total nutrient contents (NO_x, NH_4^+, and PO_4^{3-}) in the soil were determined in 1 M KCl (1:5 dilution) using a SEAL QuAAtro SFA autoanalyzer (Beun-de Ronde B.V. Abcoude, The Netherlands). Total C and N contents were determined after drying the samples in the oven at 40 °C for 5 days, and subsequently ground and sieved (0.4 mm) for the Flash EA1112 CN analyzer (ThermoFisher Scientific, The Netherlands).

DNA extraction and qPCR assay

DNA was extracted using the PowerSoil®DNA Isolation Kit (MOBIO, The Netherlands) according to the manufacturer's instruction in triplicate for each treatment. The total methanogenic population was determined for the soil, manure, and incubations with 10 % manure amendment by quantitative PCR (qPCR) targeting the *mcrA* gene using the mlas/mcrA-rev primer combination shown to capture a wide inventory of methanogens from various environments, including the cattle rumen (Steinberg and Regan 2008; Poulsen et al. 2013). The *mcrA* gene encodes for a subunit of the methyl coenzyme-M reductase and is congruent with the 16S rRNA gene phylogeny making the *mcrA* gene suitable for the detection of methanogens (Lueders et al. 2001; Luton et al. 2002). Briefly, each qPCR reaction (total volume 20 µl) consisted of 10 µl 2× SensiFAST SYBR (BIOLINE, The Netherlands), 3.5 µl of mlas forward primer (4 pmol μl^{-1}), 3.5 µl mcrA-rev reverse primer (5 pmol μl^{-1}), 1 µl bovine serum albumin (5 mg ml^{-1}; Invitrogen, The Netherlands), and 2 µl diluted template DNA. In a pilot qPCR analysis, undiluted and diluted (10×, 50×, and 100× dilution) DNA was used to determine the optimal target yield. Subsequently, template DNA was diluted 10× to obtain the optimum *mcrA* copy numbers. The PCR program consisted of an initial denaturation step at 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 25 s. Fluorescence signal was obtained at 72 °C after each cycle and melt curve obtained from 70 to 99 °C (1 °C temperature rise per cycle). Specificity of amplicon was determined from the melt curve and further confirmed by gel electrophoresis showing a single band of the correct size in the pilot qPCR run. The qPCR was performed with a Rotor-Gene Q real-time PCR cycler (Qiagen, The Netherlands). Each template DNA, from three replicated incubations, was performed in duplicate giving a total of six replicates per soil, treatment, and time.

Statistical analysis

Level of significance ($p<0.05$) between treatments was performed using one-way ANOVA as implemented in SigmaPlot

v12.5 (Systat Software Inc., USA). Error bars given as standard deviation.

Results and discussion

Methanogenic activity

The agricultural soils, including the oxic soil produced methane, but at a lower rate that the wetland soil (Figs. 1, S1, and S2). Methane was produced after an initial lag of 3–4 and 7 days in the wetland and oxic soils, respectively. The lag period was shortened to 5 days after the addition of manure in the oxic soil, but remained unchanged in the wetland soil. Therefore, the methane production rate was determined after the lag period, >4 and >7 days in the wetland and oxic soils, respectively, by linear regression (Fig. 1). Admittedly, the methane production rate determined after the lag period may not reflect on in situ rates, but was consistent with the cumulative methane production during incubation (Figs. S1 and S2). A lag before the onset of methane production suggests the occurrence of more energetically favorable redox processes (Achtnich et al. 1995) and/or growth of the methanogenic population (Fig. 2). In the incubations containing manure alone (Fig. S3), however, there was an immediate increase in methane production, suggesting of an already reduced environment harboring active methanogens. The detection of strict anaerobic methanogens and methanogenic activity in oxic soils is not without precedence; methanogens could be enriched from many oxic soils, even from a desert soil, albeit methanogenic activity was detected only after a relatively long lag period (>20 days; Peters and Conrad 1995; Angel et al. 2012).

Incubations were set up with increasing manure input (10, 20, and 40 % dry weight soil) to determine whether the response of methane production after manure amendment correlates to manure input as a source of extraneous carbon and/or methanogens. As anticipated, the addition of manure increased methane production in the short term; 10, 20, and 40 $\%_{w/w}$ manure amendment increased cumulative methane produced relative to the incubation containing only soils by approximately 3-, 3.8-, and 4.4-folds in the wetland soil and 9.5-, 30-, and 54-folds in the oxic, respectively (Figs. S1 and S2). In the wetland soil, methane production rate was not proportional to the manure input. Although statistically significant, methane production rate was not markedly higher after manure input at 20 and 40 % dry weight soil. In contrast, the amount of manure input was reflected in the methane production rate in the oxic soil, suggesting of a higher substrate limitation in this soil. Indeed, manure, a source of soluble organic C, had been shown to positively correlate to the methanogenic biomass and methane emission in agricultural soils receiving manure as fertilizer (Sommer et al. 1996; Gattinger et al. 2007; Kim et al. 2014b). Here, the manure contributed 40–175 mg excess C into the soils, depending on amendments (Table 1). In a similar incubation using the same wetland soil, we detected potential methane oxidation up to 14 μmol g dw^{-1} day^{-1} under oxic condition (Ho et al. 2013). Hence, the higher methane produced after manure amendment can be mitigated by the methanotrophs in this soil, when conditions are permissive.

Previously, we followed CO_2 emission from the rewetted gamma-irradiated rice paddy soil; CO_2 emission was not appreciable, confirming the sterility of the soil (Ho et al. 2011). Presently, we did not detect methane production in both the gamma-irradiated soils and autoclaved manure (Fig. S3). To resolve the relative contribution of the soil-borne and manure-seeded methanogens to total methane production, we performed incubations containing sterilized manure and soil, as well as manure and sterilized soil. Consistent in both soils, while the incubation with manure alone produced markedly higher methane (Fig. S3), incubation of manure in sterilized soil even after a prolonged period (16 days) showed marginal methane production (Figs. 1 and S4), indicating the relatively small contribution of manure-derived methanogens to soil methane production. Moreover, the addition of manure and sterilized manure in soils showed comparable or higher methane production rates (Fig. 1), demonstrating that the biological component in the manure was negligible for the increased methane production. In the oxic soil amended with 40 $\%_{w/w}$ sterilized manure, methane production rate was appreciably higher than amendment with manure (Fig. 1). The reason for this remains to be elucidated, but it further emphasized the importance of the physiochemical property of the manure in stimulating methane production. Moreover, the oxic soil amended with 40 $\%_{w/w}$ manure showed a different response where methane production significantly exceeded values in the incubation containing soil alone. Nevertheless, manure-derived methanogenic activity was marginal in this incubation (Figs. 1 and S2). Hence, results show a stimulation of the soil-borne methanogenic community upon manure addition, leading to increased methane production rates; manure-derived methanogens appear to play only a marginal role in the short term. In the long term, however, rumen methanogens may well contribute to the total methane production after a shift and adaptation of the methanogenic community (Rastogi et al. 2008).

Methanogen abundance assessed using the mcrA gene

We determined the potential methanogenic growth using the *mcrA* gene abundance as proxy for cell numbers in the 10 $\%_{w/w}$ manure-amended incubations. Recently, the microbial community, and more specifically, the methanogens in cattle rumen had been well-characterized (Kittelmann et al. 2013; Poulsen et al. 2013). It is not unreasonable to assume that the methanogenic community in the manure is representative

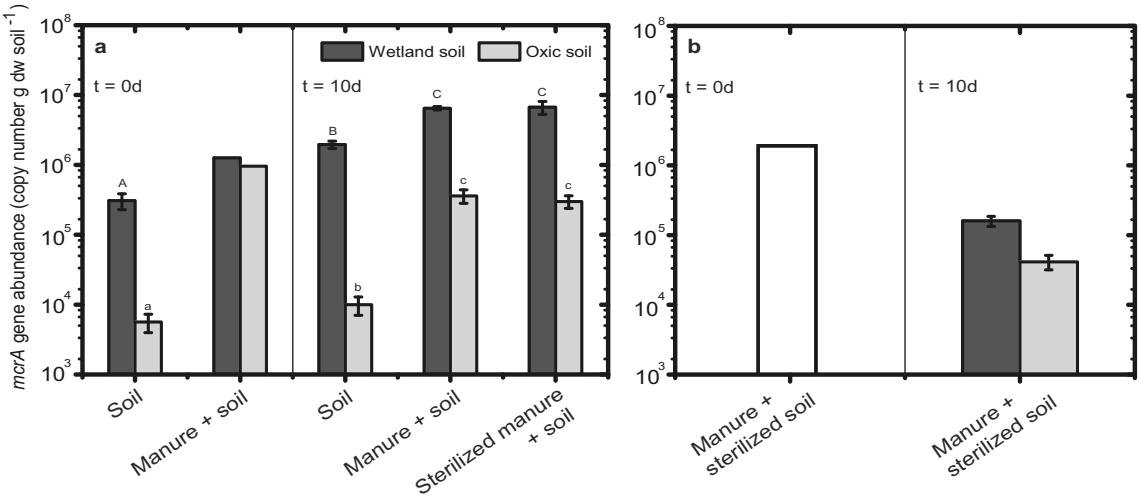

Fig. 2 Changes in the *mcrA* gene abundance (mean±s.d.; n=6) in the unamended incubation and after (autoclaved) manure amendment in the wetland and oxic agricultural soils (**a**), and in the gamma-irradiated soils amended with manure (**b**). In **a**, the initial total *mcrA* gene abundance (manure + soil) was derived by adding *mcrA* gene copies determined independently from the manure and soil. In **b**, the initial *mcrA* gene abundance was determined from the total *mcrA* gene copies detected in the manure corresponding to 1.9×10^6 *mcrA* gene copies g dw soil^{-1}. As such, these values are indicative of the starting *mcrA* gene abundance and not included in the statistical analysis. *Upper-* and *lowercase letters* indicate a significant difference at $p<0.05$ for the wetland and oxic agricultural soils, respectively

of those detected in the cattle rumen. Confirming the methane production rates, both soils harbored methanogens as indicated by the *mcrA* gene abundance with the oxic soil showing two orders of magnitude lower *mcrA* gene copy numbers (mean 5.6×10^3 *mcrA* gene copies g dw soil^{-1}) bordering the detection limit of the qPCR assay (approximately 10^4 *mcrA* gene copies g dw soil^{-1}), which reflects on the lower methane production in this soil (Fig. 2). Particularly in the wetland soil, the *mcrA* gene abundance increased significantly after amendment with (sterilized) manure, corroborating with the higher methane production rates detected (Fig. 1). In the oxic soil, the *mcrA* gene abundance was in the same order of magnitude at the initiation and after incubation despite of the manure amendment, but methane production rate was significantly higher in these incubations in comparison to values exhibited in reference incubation containing soil alone. It remains unclear whether the increase in methane production was a result of cell growth or an increase in cell-specific activity in the oxic soil. Nevertheless, integrating the qPCR analysis and process measurements from the unamended and 10 $\%_{w/w}$ manure-amended incubations for all replicates in both soils, the *mcrA* gene abundance was directly correlated to the methane production rate ($p<0.005$; Fig. S5).

Although a large population of the methanogens was seeded from the manure, they do not appear to be active in the soil immediately (up to 16 days) after amendment. This was particularly evident in incubations containing manure in gamma-irradiated soils where methanogenic activity was not detected or detected at even lower values than in incubations containing soil alone (Fig. 1), while incubations of manure alone showed immediate and markedly higher methane production (Fig. S3). The qPCR analysis was in agreement, showing that *mcrA* gene abundance decreased in incubations containing gamma-irradiated soil amended with manure after 10 days (Fig. 2). It appears that methanogens inhabiting the rumen may not be well suited to the soil environment, and presumably, were initially outcompeted by the soil-borne population.

Conclusion

Previous studies inferred that higher methane production can be attributable to the addition of methanogens via manure amendment in the field (Radl et al. 2007; Gattinger et al. 2007; Kim et al. 2014a). Despite of the methanogenic population exclusively seeded from the manure (Fig. 1; manure addition to sterilized soil), they contributed only marginally, if any, to overall immediate short-term methane production (up to 16 days) as shown in our incubations. However, in the presence of manure-dominated volumes particularly in the oxic soil, the methanogens seeded from the manure may contribute to the higher methane emission under field conditions where a high spatial heterogeneity is anticipated. Prolonged incubation may reveal the relevance of the manure-derived methanogenic community over time. More generally, we show that while rumen microorganisms are dispersed via manure, they may not show incipient activity in their new environment. Bridging our study to agriculture practice, our results emphasize the relevance of the physiochemical properties of a residue when considering methane mitigation strategies in agricultural soils.

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