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Change of the pathway of methane production with progressing anoxic incubation of paddy soil

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ABSTRACT

Rates and pathways of methane production in rice fields were found to change with season. However, field conditions are complex and thus, it is still unclear which of the many environmental factors cause these changes. One possible factor is the availability of degradable organic carbon. Therefore, we investigated the change in rate and pathway of CH_4 production under controlled laboratory conditions by progressing incubation of Italian paddy soil under anaerobic conditions. We studied the functional (pathway, rate) and structural (abundance, taxonomic composition) responses of methanogenic archaeal communities without and with addition of rice straw. Initially, rice straw significantly enhanced CH_4 production rates and acetate accumulation. Later on, the values strongly decreased with the progress of degradation. A high contribution of acetoclastic methanogenesis (62–75%) was initially observed, while hydrogentrophic methanogenesis became dominant in the late degradation phase, both in soil without and with amendment of rice straw. The percentage contribution of the hydrogenotrophic pathway scaled with the percentage of the organic carbon mineralized to CH_4 plus CO_2 during the incubation time, irrespectively whether or not the soil was amended with rice straw. It was low (< 33%), when > 60% of the carbon was still available, but increased to > 50% when available carbon had decreased to < 30%. The methanogenic archaeal communities also exhibited changes over the subsequent degradation phases, but especially when the soil was amended with rice straw. Thus, rice straw amendment initially favored acetoclastic *Methanosarcina* and hydrogentrophic *Methanocella*, which subsequently decreased, while acetoclastic *Methanothrix* ('*Methanosaeta*') was favored and increased in the late degradation phases. The results demonstrate strong functional and structural responses of methanogenic archaeal communities to progressing degradation of rice straw and/or soil organic matter, but indicate that the hydrogenotrophic methanogenic pathway was in particular controlled by the availability of degradable organic carbon.

1. Introduction

Rice fields are one of the major sources in the global methane budget and contribute in the range of 25–300 Tg CH_4 per year (Bridgham et al., 2013; Chen and Prinn, 2005). Therefore, the processes involved in CH_4 formation in rice fields are of great interest. The CH_4 is usually produced from either acetate or H_2/CO_2, the two major products of organic matter fermentation (Conrad, 1999, 2007). Methane production involves a rather large diversity of methanogenic archaea, including acetoclastic *Methanosarcinaceae* and *Methanotrichaceae* ('*Methanosaetaceae*') (Oren, 2014) as well as hydrogenotrophic *Methanocellales*, *Methanomicrobiales*, and *Methanobacteriales* (Bao et al., 2016; Conrad et al., 2012; Lu et al., 2015). Hydrogenotrophic methanogenesis strongly prefers the isotopically lighter carbon, whereas a smaller kinetic isotope effect is expressed in acetoclastic methanogenesis (Whiticar et al., 1986), in which CH_4 is almost exclusively produced from the methyl group of the acetate (Stadtman and Barker, 1951). This difference in isotopic fractionation can in principle be used to calculate the relative contribution of the two methanogenic pathways to total CH_4 production (Conrad, 2005). Alternatively, the contribution of hydrogenotrophic methanogenesis has been determined by labelling with radioactive bicarbonate (Conrad and Klose, 1999), by specific inhibition of acetoclastic methanogenesis with methyl fluoride (Conrad and Klose, 1999), and by quantification of fermentation products upon inhibition of CH_4 production (Glissmann and Conrad, 2000).

The relative contribution of hydrogenotrophic and acetoclastic methanogenesis, i.e. the pathway of CH_4 production, systematically changes upon flooding of paddy soil. The reason is the progressive

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expression of fermentation reactions, which convert organic matter to the methanogenic precursors H_2, CO_2 and acetate, as well as the competition of ferric iron-reducing and sulfate-reducing microorganisms with methanogens for these substrates (Glissmann and Conrad, 2002; Roy et al., 1997; Yao and Conrad, 1999). When inorganic electron acceptors such as ferric iron and sulfate are completely reduced, CH_4 production becomes the exclusive final step in mineralization of organic matter. Then, fermentation of organic matter and CH_4 production eventually achieve quasi-steady state at which the methanogenic substrates (H_2, acetate) exhibit a low concentration, and organic matter (e.g. polysaccharides) is converted to equal amounts of CO_2 and CH_4 with a contribution of about < 33% hydrogenotrophically and > 67% acetoclastically produced CH_4 (Conrad, 1999). Such conditions are on the average (n = 16; Yao et al., 1999) reached about 20 days after submergence, depending on the ratio of available organic matter to inorganic electron acceptors (mainly ferric iron) (Yao et al., 1999) and the temperature (VanBodegom and Stams, 1999; Yao and Conrad, 2000).

The temporal change of the methanogenic pathway has frequently been studied under field conditions. A rice growing season typically lasts for 90–120 days, i.e., much longer than the average lag time (about 20 days) for initiation of methane production after flooding (Yao et al., 1999). Methanogenic pathway data under field conditions are quite varied. Most studies found dominance of acetoclastic methanogenesis throughout the rice growing season (Krüger et al., 2001; Nakagawa et al., 2002b; Tyler et al., 1997), while other studies found this dominance preferentially late in the season (Krüger et al., 2002; Zhang et al., 2011). However, field conditions are complex, since CH_4 is produced from three different sources of organic matter, i.e., root exudates, straw, and soil organic matter (SOM), whose relative contributions change with time of the season (Watanabe et al., 1999; Tokida et al., 2011; Yuan et al., 2012). While rice straw is a major source early in season, root exudates become increasingly important with the development of rice plants (Watanabe et al., 1999; Tokida et al., 2011; Yuan et al., 2012). The rice plants also affect the transport of CH_4 from the soil into the atmosphere since their aerenchyma serves as conduit for gas exchange, also affecting the isotopic composition of the CH_4 (Chanton et al., 1997; Conrad and Klose, 2005). In addition, processes are affected by oxygen which diffuses through the rice aerenchyma into the rhizosphere and results in partial oxidation of the produced CH_4, including effects on its isotopic composition (Bilek et al., 1999; Krüger et al., 2002; Tyler et al., 1997; Zhang et al., 2016). Thus the methanogenic pathway usually cannot be determined unambiguously under field conditions (Krüger et al., 2002; Tyler et al., 1997; Zhang et al., 2016). Therefore, we decided to investigate the pathway of CH_4 production under laboratory conditions, when only SOM and rice straw were available as sources of organic matter, and conditions were completely anoxic and reduced. We hypothesized that the pathway of CH_4 production is affected by the availability of organic matter and that the hydrogenotrophic pathway will increase with progressing degradation of organic matter. This hypothesis was based on observations in deep lake sediments (Chan et al., 2005; Conrad et al., 2010, 2011; Liu et al., 2017; Lofton et al., 2015) and permafrost soil (Hodgkins et al., 2014; Nakagawa et al., 2002a), where the hydrogenotrophic pathway increased with sediment depth and age of organic carbon being indicative for increased recalcitrance.

We determined methanogenic function by rates and pathways of CH_4 production, and characterized the methanogenic community structures by the abundance and taxonomic composition of the *mcrA* gene, which codes for a subunit of the methyl coenzyme M reductase, the key enzyme of methanogenesis. The experiments were based on repeated incubation of Italian paddy soil with and without amendment of rice straw and recording of the temporal change in total gaseous C production as measure of available organic matter.

2. Experimental procedures

2.1. Soil incubation and chemical analyses

Soil was sampled from rice fields at the Italian Rice Research Institute in Vercelli, Italy, in 2013 and was air dried and stored at room temperature. The soil was sieved (< 2 mm) prior to use. Rice straw was prepared by growing rice plants in the greenhouse using Italian rice field soil (Pump and Conrad, 2014). The straw was air-dried and ground using a blender. The physicochemical properties of soil and rice straw are described in Table S1.

The incubation procedure has been described before (Conrad et al., 2011). Soil slurries were prepared by mixing 600 g dry soil with 600 mL of deionized, sterile, anoxic water, and incubating the mixture in 2-L bottles under a headspace of N_2 at 25 °C for 2 weeks of pre-incubation. After pre-incubation, two treatments were established by dispensing the slurries into 200-mL bottles, with three replicates each of unamended soil (SOIL) and of soil amended with rice straw (RS) at a ratio of 5 mg dry ground straw per gram dry soil. Immediately after RS addition, the bottles were sealed again and flushed with N_2 and, after shaking, re-flushed with N_2 to remove the residual O_2 and CH_4. Then the bottles were incubated statically at 25 °C for 120 days. At 0, 30, 60, 90 and 120 days, aliquots (10 g slurry) from the two treatments were dispensed into 26-mL pressure tubes, the time points corresponding to five phases of progressive decay of SOM and rice straw (D0, D30, D60, D90, D120). The tubes were closed with black rubber stoppers, flushed with N_2, pressurized to 0.5 bar overpressure, and incubated at 25 °C for about 20–30 days. The tubes with soil slurry were prepared in numerous parallels, of which triplicates were sacrificed for chemical analyses. The gas phase of some incubations was amended with 2% CH_3F, an inhibitor of acetoclastic methanogenesis (Conrad and Klose, 1999; Janssen and Frenzel, 1997). Gas samples were taken during the course of the incubation and analyzed for CH_4, CO_2 and $\delta^{13}C$ of CH_4 and CO_2 at 2–3-day intervals. In the end, the tubes were opened and samples retrieved for analysis of acetate and $\delta^{13}C$ of acetate. Soil samples were taken from the triplicate treatments SOIL and RS in the beginning of the five phases (D0, D30, D60, D90, D120) and stored frozen at −20 °C for molecular analysis.

The chemical analysis of gas and liquid samples was carried out as described before (Conrad et al., 2011). Briefly, CH_4 and CO_2 were analyzed by gas chromatography (GC), acetate by high-pressure liquid chromatography (HPLC) and the $\delta^{13}C$ by either GC combustion isotope ratio mass spectrometry (GC-C-IRMS) or HPLC-C-IRMS. The $\delta^{13}C$ of the methyl group of acetate was determined after off-line pyrolysis. The $\delta^{13}C$ of organic matter was analyzed by the Centre for Stable Isotope Research and Analysis (KOSI) at the University of Göttingen, Germany, using an elemental analyzer coupled to an IRMS.

The time courses of CH_4 accumulation starting from day 6 in each phase were used to calculate production rates of treatments SOIL and RS. The average $\delta^{13}C$ values of CH_4 and CO_2 were determined from day 12 to day 24 in each phase for each replicate from treatments SOIL and RS. The $\delta^{13}C$ of the methyl group of acetate was analyzed only at the end of each incubation. These data from treatments SOIL and RS were then used for calculating the relative contribution of hydrogenotrophic methanogenesis to total CH_4 production (f_{H2}) using the mass balance from the $\delta^{13}C$ of CH_4 in the presence ($\delta^{13}C_{CH4\text{-}mc}$) and absence ($\delta^{13}C_{CH4}$) of CH_3F and from the $\delta^{13}C$ of the methyl group of acetate ($\delta^{13}C_{ac\text{-}methyl}$) as described in detail before (Conrad et al., 2011): $f_{H2} = (\delta^{13}C_{CH4} - \delta^{13}C_{ac\text{-}methyl})/(\delta^{13}C_{CH4\text{-}mc} - \delta^{13}C_{ac\text{-}methyl})$.

For determination of the available amounts of organic carbon, total gaseous products (gaseous CH_4 + total CO_2) were determined in the five phases (D0, D30, D60, D90, D120), by measuring the partial pressures of CH_4 and CO_2 in the headspace of the tubes. Total CH_4 was determined from the amount in the gaseous headspace neglecting the small amounts of dissolved CH_4. Total CO_2 was determined from both the gaseous and dissolved CO_2 plus the amount dissolved as