Applied Soil Ecology 119 (2017) 267–274

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Methane oxidation and diversity of aerobic methanotrophs in forest and agricultural soddy–podzolic soils

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ARTICLE INFO

Keywords:
Atmospheric CH_4 oxidation
Forest soils
Uncultured methanotrophs
pmoA

ABSTRACT

Soddy-podzolic soils are widely distributed in European Russia, but their role as a sink for atmospheric methane is poorly documented and there is no information on the methanotroph diversity. We analysed the potential CH_4-oxidation rates in soil samples and showed that the rate was significantly higher in forest soil than in arable soil, 1.21 and 0.40 ng CH_4 g soil $^{-1}$day^{-1}, respectively. PCR-DGGE and clone library analysis indicated the distinct methanotrophic communities in these soils. The *pmoA* sequences associated with uncultured soil methanotrophs, referred to as NUSC, dominated forest soil, while in agricultural soil, type I (*Methylobacter, Methylocaldum*) and type II (*Methylocystis, Methylosinus*) methanotrophs were dominant. A newly developed primer set was applied in qPCR analysis and revealed that the copy number of *pmoA* genes of NUSC methanotrophs in forest soil was $(9.2 \pm 0.87) \times 10^4$ g soil^{-1}, whereas the transcript number was $(1.33 \pm 0.31) \times 10^6$ g soil $^{-1}$. We concluded that differences between the CH_4 oxidation rates between forest and agriculture soils were driven by the structure of the methane-oxidizing community and that a novel group of methanotrophs may be an active participant in this process.

1. Introduction

Methane (CH_4) is an important trace gas, and its contribution to the greenhouse effect is estimated as high a 30% (Dlugokencky et al., 2011). The only recognized biological mechanism of regulation of methane content in Earth's atmosphere is its oxidation by microbial communities of upland soils, up to 30 Tg year −1 (Denman et al., 2007). Many studies have investigated CH_4 uptake in the soils of natural ecosystems and have found them to be the sink for atmospheric methane (Börjesson et al., 2001; Conrad and Rothfuss, 1991; Suwanwaree and Robertson, 2005). Conversion of natural undisturbed soils to arable cropping ecosystems has significantly reduced the CH_4-oxidising capacity of these soils (Le Mer and Roger, 2001). Agricultural practices also affect methanotrophic community structure (Knief et al., 2003; Seghers et al., 2003; Kravchenko et al., 2005). Biological methane oxidation is important for minimizing global climate change, and any negative impact or imbalance may be due to dramatic ecosystem change. Therefore, there is an insistent need for extensive research to study methanotrophic activity in various ecosystems.

Methane oxidation is performed by methane oxidizing bacteria (MOB), which use methane both in energetic and constructive metabolism to the end products carbon dioxide and water (Hanson and Hanson, 1996).The methanotrophs are a subgroup of the methylotrophs and are generally characterized by their ability to use methane as their sole carbon and energy source (Hanson and Hanson, 1996). The key methanotrophic enzyme is methane monooxygenase (MMO), which occurs in both particulate (pMMO) and soluble (sMMO) forms. The *pmoA* gene encodes the β-subunit of pMMO and is included in the genome of the majority of known methanotrophs, except *Methylocella* and *Methyloferula* (Dedysh et al., 2000; Vorobev et al., 2011**).** For a long period, all methanotrophs were affiliated with *Proteobacteria* from the *Methylocystaceae* and *Methylococcaceae* families (Hanson and Hanson, 1996), but new aerobic methanotrophs were found in *Gammaproteobacteria* (*Methylococcaceae*, *Crenothrix polyspora*, and *Clonothrix fusca*), *Alphaproteobacteria* (*Methylocystaceae* and *Beijerinckiaceae*), the *Verrucomicrobia* phylum ("*Methylacidiphilaceae*") and the candidate phylum NC10 (Stein et al., 2012). Like most other aerobic methane oxidizers, methane-oxidizing *Verrucomicrobia* (*Methylacidiphilum* and *Methylacidimicrobium*) use pMMO to catalyze the first step of the methane oxidation, but unlike most proteobacterial methanotrophs grow as autotrophs, using only carbon dioxide as the carbon source via the Calvin cycle (van Teeseling et al., 2014). The novel phylum NC10 represents bacteria capable of aerobic methane oxidation coupled to denitrification under anoxic conditions (Ettwig et al., 2010). In addition, a group

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http://dx.doi.org/10.1016/j.apsoil.2017.06.034
Received 13 February 2017; Received in revised form 23 June 2017; Accepted 28 June 2017
Available online 12 July 2017

of methanogen-like anaerobic CH_4-oxidizing archaea (MOA) has been described (Hallam et al., 2003). These MOA contain *mcrA* genes and are involved in a consortium that couples denitrification with anaerobic CH_4 oxidation (Raghoebarsing et al., 2006). Moreover, ammonia oxidizers were also shown to be able to convert methane to methanol using an enzyme homologous to the methane monooxygenase of methanotrophs. However, it appears that they cannot grow using this process (Hyman and Wood, 1983; Jones and Morita, 1983). Until 2005, methanotrophs were regarded as organisms whose growth was obligatorily one-carbon compound-utilizing, but it was reported that *Methylocella* can utilize multi-carbon compounds besides methane (Dedysh et al., 2005). *Crenothrix polyspora*, a sheathed γ-Proteobacteria, was identified as another possible candidate facultative methanotroph (Stoecker et al., 2006). More recently, a pMMO-possessing methanotroph of the genus *Methylocapsa*, as well as some *Methylocystis* species, were demonstrated to be able to grow on acetate as the sole substrate (Belova et al., 2011; Dunfield et al., 2010). The facultative lifestyle in methanotrophs indicates that broader substrate utilization might be more common in methanotrophs than previously thought.

Methane uptake in upland soils is mediated by aerobic methane-oxidizing bacteria, which are the only known biological sink of CH_4. Despite the active study, the ecological representativeness of the data available for methane oxidation in aerated soils is insufficient. It is especially important to understand the role of Russian soils, which are not usually included in the general reviews of atmospheric methane uptake in soil (Kirschke et al., 2013; Serrano-Silva et al., 2014) or soil methanotroph diversity (Aronson et al., 2013) due to the lack of published data. The majority of known aerobic methanotrophs are not capable of using low-concentration atmospheric methane. Some representatives of *Methylocystis* and *Methylosinus* showed the presence of two isoforms of methane monooxygenase—conventional MMO1 and high-affinity MMO2 (Baani and Liesack, 2008; Kravchenko et al., 2010; Belova et al., 2013). The culture-independent studies of methanotrophic communities of soils with high-affinity methane oxidation capacity have revealed their presence and a correspondingly frequent predominance of methanotrophs from the novel phylogenetic *pmoA*- lines from *Alpha*- and *Gammaproteobacteria* named USC-alpha (upland soil cluster) and USC-gamma (Knief et al., 2003; Kolb et al., 2005). USC-alpha bacteria are related to *Methylocapsa acidiphila* (Ricke et al., 2005) and USC-gamma bacteria are distantly related to *Methylococcaceae* (Knief et al., 2003). Cluster I, another rooted phylogenetic branch of uncultured methanotrophic *Alphaproteobacteria,* was found in forest soils (Ricke et al., 2005). Uncultured methanotrophs forming a compact phylogenetic cluster were found in virgin forest and steppe soils (Kizilova et al., 2013). Uncultured methanotrophs are responsible for atmospheric methane oxidation, as shown by stable tracer investigations (Bengtson et al., 2009; Menyailo et al., 2010). To date, there are no any culturable high-affinity methanotrophs, so data regarding their phylogeny and participation in atmospheric methane oxidation are elusive.

In this study we analysed potential methane oxidation rates under laboratory conditions, and also assessed the diversity of aerobic methanotrophs using a culture-independent approach consisting of amplification and cloning of *pmoA* gene fragments in forest and arable soddy-podzolic soil samples. We hypothesized direct connections between the shifts in the microbial communities and the rates of methane oxidation activity.

2. Materials and methods

2.1. Soil characteristics and sampling

For this study, we chose two experimental sites at the Timiryazev Agricultural Academy, Moscow, Russia (55°49′ N., 37° 32. 24′ E). One experimental site (M1) was in a temperate mixed forest (*Pinus* spp., *Abies* spp., *Betula* spp.) inside the Forest Experimental Station and the other (M2) in a permanent barley (*Hordeum vulgare*) crop plot without fertilization inside the Long-term Field Experiment established by A.G. Doyarenko in 1912 on soddy-podzolic soils (Table 1). Soddy-podzolic soils (podzoluvisols according to FAO classification) are typical for a mixed forest zone from the 54–58° to 60° northern latitude and cover approximately 70% of the Moscow region's territory. The mean temperatures of the coldest and warmest months are 2–14 and 9.5–16.5 °C, respectively, and the amount of annual precipitation ranges from 500 to 700 mm. The growing season varies from 120 to 174 days. Soddy-podzolic soils are highly acidic (pH 3.5–5.5) with low base saturation, weak aggregation, considerable amounts of crude plant residue, and a predominance of brown humus. Preliminary studies from these sites indicated the surface CH_4 uptake in the forest and the arable soil, respectively (Chistotin et al., 2012) (Table 1).

On September 15, 2014, soil samples (0–20 cm) were collected from five points for each site, one in the centre and four at the corners of a 20 m × 20 m plot, and shipped to the lab in a cooler. Fresh soil samples were sieved (2 mm) and stored at 4–6 °C in aerated plastic bags before being analysed for potential methane oxidation. DNA and RNA extraction was performed immediately upon arrival to the laboratory (about three h) and stored at −80 °C until required for analysis. Both soils were characterized as deep soddy medium podzolic soils with a loamy, sandy texture (40% sand, 46% silt, and 14% clay) free of carbonate; the pH (1 M KCl) was 5.3 in the arable site and 4.2 in the forest site.

2.2. Potential rate of methane oxidation

A radioisotope tracer technique with ^{14}C-labeled methane was applied for evaluation of potential methane oxidation activity and assimilation processes according to a previously described protocol (Kravchenko et al., 2005). Briefly, 50 µl of an aqueous solution of $^{14}CH_4$ (0.08 MBq; Izotop, Russia) was added to 5 g soil samples (fresh weight) in 20 ml Hungate tubes, and the final concentration was approximately 10 nL mL^{-1} (1.3 nmol CH_4 g^{-1} or 10 ppm). After 72 h incubation at room temperature, soil samples were fixed in 2 ml of 1 N KOH. The separation and assays of ^{14}C products were performed according a previously described protocol (Rusanov et al., 1998). $^{14}CH_4$ was burned to $^{14}CO_2$ in an oven over a catalyst ($CoCl_2$-impregnated silica gel). $^{14}CO_2$ was captured in two traps (assembled before and after the oven with catalyst) containing a 10% solution of 2-phenylalanine in toluene scintillation liquid GS-106 (Monokristall, Ukraine). Upon removal of volatile products, the ^{14}C content of organic matter was determined by the method of "wet" burning to $^{14}CO_2$ in the presence of $K_2S_3O_8$ at 105 °C. Radioactivity was measured with a RackBeta 1219 liquid scintillation counter (LKB, Sweden). Roughly, we considered the following parameters: amount of radioactive carbon dioxide, which formed during microbial oxidation of $^{14}CH_4$, incorporation of ^{14}C into

Table 1
Selected soil characteristics and field methane fluxes of studied sites. Shown are the mean values and standard deviations.

Soil ID	Organic C, %	Total N, %	C:N	pH (1 M KCl)	NO_3^- + NH_4^+, (µg g^{-1})	16 s rRNA, (10^8 gene copies g^{-1})	*pmoA* (10^5 gene copies g^{-1})	CH_4 flux,[a] (µg C m^{-2} h^{-1})
M1	2.1 ± 0.3	0.2 ± 0.04	10.5	4.6 ± 0.05	8.4 ± 0.2	10.57 ± 1.26	2.83 ± 0.25	−19.0 ± 3.4
M2	1.2 ± 0.2	0.1 ± 0.02	11.9	5.3 ± 0.05	8.3 ± 0.2	8.64 ± 1.25	0.46 ± 0.08	−2.6 ± 1.2

[a] Chistotin et al. (2012).