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EXPERIMENTAL ARTICLES

Physicochemical and Biological Factors Affecting Atmospheric Methane Oxidation in Gray Forest Soils

I. K. Kravchenko*, V. M. Semenov, T. V. Kuznetsova**, S. A. Bykova*, L. E. Dulov*, D. Pardini***, M. Gispert***, P. Boeckx****, O. Van Cleemput****, and V. F. Gal'chenko***

**Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

***Institute of Physicochemical and Biological Problems of Soil Science, Russian Academy of Sciences, Pushchino, Moscow oblast, Russia*

****University of Girona, Spain*

*****Ghent University, Belgium*

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Abstract—The decline of methane oxidizing activities in gray forest soil upon its conversion into arable land was shown to be caused by major changes in biotic and physicochemical properties of soil. Using the method of immune serums, methane-oxidizing bacteria were detected in both forest and agricultural soils, but their populations differed significantly in both abundance and composition. In the forest soil, the number of methanotrophs was an order of magnitude higher than in arable soil, amounting to 3.5×10^8 and 0.24×10^8 cells/g soil, respectively. All methane-oxidizing bacteria identified in the forest soil belonged to the genus *Methylocystis*, and 94% of these were represented by a single species, *M. parvus*. The arable soil was dominated by type I methanotrophs (*Methylobacter* and *Methylomonas*, 67.6%), occurring along with bacteria of the genus *Methylocystis*. In addition, arable soil is characterized by a low content of microbial biomass, lower porosity and water resistance of soil aggregates, and the predominance of nitrogen mineralization processes over those of nitrogen immobilization. These factors can also contribute to lower rates of methane oxidation in arable soil as compared to forest soil.

Key words: methane-oxidizing bacteria, forest and arable soils.

Serving as a sink for atmospheric methane, aerated soils play an important role in the biogeochemical methane cycle and, for that matter, contribute to global climate change. Uptake of atmospheric methane was shown in soils of forest and tundra ecosystems [1, 2]; soils of anthropogenically changed ecosystems, including arable soils; soils of landfills; and drained fenland peat [3, 4]. The methane-oxidizing capacity of boreal zone soils is known to decline significantly as they are turned into arable land [5], when nitrogen fertilizers are applied, or as a result of contamination by the atmospheric nitrogen compounds [6]. In our recent study of seasonal variation of atmospheric methane input into gray forest soil of the Moscow region, the methane fluxes were shown to be significantly higher in forest biocenoses than in agrocenoses [7].

Despite a large number of studies devoted to atmospheric methane uptake in aerobic soils, the mechanisms that control this process are not yet well understood. Based on studies of methane oxidation kinetics, it was hypothesized that the rate of this process could depend on the microbial community composition [8], variations in the processes of carbon and nitrogen mineralization [9], and the aeration regime [10]. At the same time, we are not aware of any experimental studies that actually tested these hypotheses.

The goal of this work was to carry out a comparative study of physicochemical and biological parameters that, in our view, could control the capacity of gray forest soil to oxidize atmospheric methane in forest and agricultural ecosystems.

MATERIALS AND METHODS

Object of study. The studies were carried out at the "Forest" and "Agrocenosis" test plots, Field Research Station of the Institute of Physicochemical and Biological Problems of Soil Science, Russian Academy of Sciences, Pushchino, Moscow oblast. The test sites were described in detail elsewhere [7].

Methane oxidation kinetics was studied in laboratory incubation assays. Samples of soil (10 g) were placed in 120-ml bottles. The initial methane concentration in the gas phase was 2.0–3.3 nl CH_4/ml (that of ambient air in the laboratory). In the course of 120 h, air samples (0.5 ml) were taken from the headspace of the bottles every 8–24 h and analyzed on a Kristall 5000.1

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gas chromatograph (Russia) equipped with a flame-ionization detector (column length, 2 m; diameter, 2 mm; Chromosorb 102, 80/100 mesh as the sorbent; nitrogen as the gas carrier; gas flow rate, 30 ml/min; column temperature, 40°C; the evaporator and detector temperatures, 100°C). The time variation of the methane concentration was described by the equation $d[CH_4]/dt = k_1x[CH_4]$. The rate of methane oxidation in soil was calculated as a product of the rate constant k_1, h^{-1} and the initial methane content of the gas phase.

The intensity of methane oxidation and assimilation processes was determined by the radioisotopic method [11]. Samples of soil (5 g) were placed in bottles and 50 µl of water solution of radioactively labeled methane ($^{14}CH_4$, Izotop, Russia) was then introduced in each bottle to make the methane concentration in the headspace approximately equal to 10 nl/ml. The bottles were incubated for 48 h at the in situ temperature and then fixed with 2 ml of 1 N KOH.

The separation and assays of ^{14}C products formed were carried out as described elsewhere [11]. $^{14}CH_4$ 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).

The number of bacteria in soil was determined by means of luminescent microscopy [7]. The species composition of methanotrophic bacteria was determined and enumerated by the method of indirect immunofluorescence [12]. Polycarbonate filters with precipitated bacteria were treated with immune serums specific to 12 species of methanotrophic bacteria, and the number of individual species was counted under a microscope. The overall number of methanotrophs was determined as a total of individual identified species.

Analytic assays. All chemical assays were performed in four replicates. The contents of NH_4^+–nitrogen and NO_3^-–nitrogen were determined by the phenolate hypochlorite method directly in soil extract and after reducing nitrates to ammonium with zinc powder and 10% solution of $CuSO_4$. Organic carbon and nitrogen were assayed by the Tyurin and the Kjeldahl method, respectively. The porosity and structural stability of soil aggregates were determined at the University of Girone, Spain. The total porosity and pore diameter were determined using a Fisons 200 mercury porosity meter with 140 cells operating under Milestone software. The method of Greenland was employed to classify soil aggregates on the basis of a water coherence test [13].

Table 1. Major physical, chemical, and water-physical properties of gray forest soil

Parameter	Forest	Agrocenosis
Granulometric composition:		
sand, %	1.8 ± 0.5	2.3 ± 0.9
silt, %	14.8 ± 0.5	14.2 ± 1.0
clay, %	37.6 ± 0.7	40.6 ± 1.9
C org, %	1.82 ± 0.04	0.96 ± 0.10
N total, %	0.15 ± 0.03	0.10 ± 0.02
pH	5.18 ± 0.03	6.50 ± 0.02
C in microbial biomass, mg/100 g	158 ± 3	33.4 ± 3.1
N in microbial biomass, mg/100 g	38.0 ± 1.1	10.3 ± 0.4
$N–NH_4$, mg/100 g	0.29 ± 0.04	0.10 ± 0.01
$N–NO_3$, mg/100 g	0.27 ± 0.03	0.49 ± 0.06
Porosity, %	55.8 ± 1.41	43.5 ± 5.34
Mean pore diameter, µm	2.17 ± 0.33	1.84 ± 0.35
Fraction of dispersed soil aggregates, %	15.0 ± 3.5	93.8 ± 1.8
Soil aggregate structural stability class	7	2

Statistical analysis of the obtained data was done using Microsoft Excel under Windows XP.

RESULTS AND DISCUSSION

Although the methane flux from the atmosphere into gray forest soil showed considerable seasonal variation, its mean annual value for the unused woodland plot "Forest" was more than three times greater than for the farmed plot "Agrocenosis" [7]. Based on published evidence and results of our previous studies, it was hypothesized that the reduction of methane oxidation activity in arable soil was due to significant and irreversible changes both in the population structure of its methanotrophic communities and in its physicochemical properties that control this activity.

Persistent exposure to agricultural practices entailing, among other things, change in local environmental conditions with the change in vegetation can be seen to alter the physicochemical and water-physical properties of arable soils as compared to intact soils (Table 1). Protracted use of gray forest soil for agricultural production led to a 1.9-fold decline of organic carbon content and a 1.5-fold reduction of total nitrogen, with nitrate becoming a predominant mineral form. Elsewhere, we showed that processes of atmospheric methane oxidation and nitrogen transformation were closely linked [9]. The pH value changed from mildly acidic (5.18) in forest soil to almost neutral (6.50) in arable soil and the total microbial biomass in soil decreased significantly, as indicated by lower values of microbial

Table 2. Species composition of methanotrophic communities in gray forest soil of Moscow oblast determined by immune serum assays

	Forest biocenosis			Agrocenosis		
	10^6 cells/g	% of the total number	% of all methanotrophs	10^6 cells/g	% of the total number	% of all methanotrophs
Methylocystis echinoides^T	5.2	0.1	1.5	1.3	0.05	3.8
"*Methylocystis methanolicus*"^{T}	–	–	–	4.9	0.20	14.3
"*Methylocystis minimus*"^{T}	15.4	0.3	4.4	1.9	0.08	5.7
"*Methylocystis pyriformis*"	–	–	–	1.0	0.04	2.9
Methylocystis parvus^T	329.4	6.7	94.1	1.9	0.08	5.7
Methylosinus sporium^T	–	–	–	–	–	–
Methylosinus trichosporium^T	–	–	–	–	–	–
Methylomonas methanica	–	–	–	3.6	0.14	10.5
"*Methylobacter vinelandii*"	–	–	–	19.4	0.8	57.1
"*Methylobacter bovis*"^{T}	–	–	–	–	–	–
"*Methylobacter chroococcum*"^{T}	–	–	–	–	–	–
Methylococcus capsulatus	–	–	–	–	–	–
Total number of bacteria	4940.9			2462.0		
Total number of methanotrophs	350.0	7.1		34.0	1.38	

Note: "–" means not found.

carbon and nitrogen contents (Table 1) and by the reduction of the overall number of bacteria (Table 2).

The key physical property of soil controlling the balance between aerobic and anaerobic processes and gas diffusion is its porosity. In the forest soil, it amounted to 55–57%, ensuring favorable conditions for water and air permeability (Table 1). Agricultural use of soil results in overcompactness of its tillable layer and causes its porosity to drop down to 40–47%. The effect of a marked decrease in the mean pore diameter in arable soil should be taken into account. In the forest soil, pores were mostly large (300–30 μm) and medium sized (30–3 μm), whereas, in the agrocenosis soil, the diameter of most of pores was under 3 μm (Table 1). Soil aggregates in the agrocenosis soil had low stability and were virtually disintegrated by wetting (Table 1). A drastic deterioration of gas permeability of soil as a result of both the predominance of fine and inactive pores and the low water resistance of soil aggregates is likely to be one of the causes of decreased methanotrophic activity in cultivated soil.

Our incubation experiments in the laboratory revealed a significant difference in the capacity of "Forest" and "Agrocenosis" soil samples to oxidize methane at low concentrations that were similar to those in the atmosphere (see figure). In samples of forest soil, the concentration of methane in the headspace of bottles declined tenfold after an 8- to 12-h lag phase and stabilized at a level of 0.4–0.6 nl/ml after 72 h of incubation. The rate of methane oxidation was estimated to be 0.7 ± 0.03 ng CH_4/(g h), in agreement with the results obtained for forest soils in Europe by other authors [14]. By assuming the weight of the active methane-oxidizing soil layer to be roughly 80 kg/m^2 (layer thickness, 10 cm; specific weight, 1 g/cm^3; moisture, 30%), one finds that the flux of atmospheric methane into the soil of the "Forest" test plot could amount to 1.34 mg CH_4/(m^2 day). The introduction of acetylene in the headspace of bottles, which inhibits the activity of microbial monooxygenases, was found to eliminate the methane-oxidizing activity in samples of forest soil (see figure), thus attesting to the bacterial nature of this process.

In tests with samples of arable soil, no reliable change in the concentration of methane in the headspace of bottles was observed during 72 h of incubation. Determination of the oxygen content in the gas phase showed that it could not be a limiting factor for oxidation of methane.

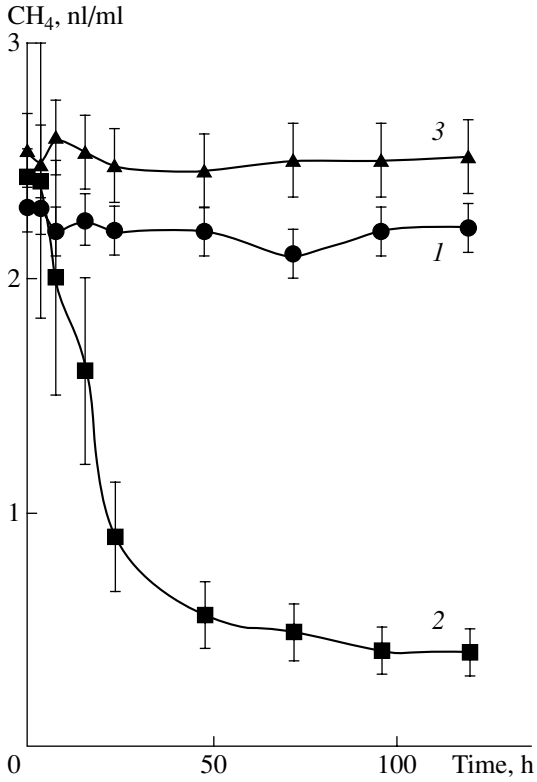
The results of radioisotopic experiments confirmed that the rate of atmospheric methane oxidation in arable soil (0.02 ng CH_4 carbon/(g day)) was much lower than in forest soil (0.32 ng CH_4 carbon/(g day)). At the same time, the distribution of ^{14}C between CO_2 and the microbial biomass and metabolites turned out to be fairly similar in both types of soil. The main product of bacterial oxidation of methane was carbon dioxide, accounting for more than 60% of ^{14}C introduced in either forest or arable soils. The organic matter, biomass and exometabolites, in the forest and arable soils incorporated, respectively, 33 and 34% of the introduced ^{14}C. Such a distribution of methane carbon dif-

fers from that observed in the energy and constructive metabolism of methanotrophs growing under balanced conditions, when up to 50% of methane carbon is revealed in CO_2 [15]. Also the percentage of methane carbon incorporated in organic matter in our tests was much higher than that observed in West Siberia soils, where it never exceeded 5–8% [11].

The obtained evidence indicated significant distinctions in the composition of methane-oxidizing communities in the intact forest biocenosis and the agrocenosis studied. This hypothesis was experimentally tested by analyzing the species composition of the methanotrophic communities in these soils by the method of immunofluorescence.

The forest and arable soils alike were found to contain widespread methane-oxidizing species, but their total abundances and species compositions differed significantly. In the "Forest" test plot soil, *Methylocystis parvus* dominated absolutely among methanotrophic bacteria, accounting for 94% of the total number of identified methanotrophs (Table 2). In the arable soil, the abundance of this species was much lower, constituting less than 6% of the total number of methanotrophs. By contrast, the number of *Methylobacter vinelandii* in the forest soil was under the detection threshold, whereas, in the arable soil, this species dominated and accounted for 57.1% of the net population of methanotrophs. The total number of methanotrophs in samples of forest soil was roughly ten times higher than that in arable soil. These figures are in agreement with the evidence obtained by British scientists who estimated, in plating experiments, the most likely abundance of methanotrophs in forest and arable soils of Great Britain [16]. It is worth emphasizing that the methane-oxidizing community in soil of the forest biocenosis was fairly homogeneous, being represented by species of the genus *Methylocystis* alone (group II methanotrophs). The arable soil community proved to be far more diverse, containing not only identified representatives of the genus *Methylocystis* but also those belonging to group I methanotrophs *Methylomonas* and *Methylobacter*, which accounted for 66.7% of the total methanotrophic population.

Bacterial oxidation in soil is the sole biological sink for atmospheric methane. The oxidation of atmospheric methane in forest, arable, and other aerobic soils was earlier proven convincingly to be connected with the activity of methanotrophic bacteria. At the same time, it is not known which methanotrophs carry out this process. Based on studies of methane oxidation kinetics, Bender and Conrad suggested that, in addition to methane-oxidizing bacteria with low substrate affinity (micromolar values of K_m), there must exist so far unknown high-affinity methanotrophs (nanomolar values of K_m) responsible for methane oxidation at very low atmospheric concentrations [17]. This hypothesis was substantiated in studies of microbial communities of aerobic soils carried out with the use of *pmoA* gene analysis [18] and radioactive fingerprinting [19], which revealed the occurrence of unknown methanotrophs belonging to the α-group of *Proteobacteria.* On the other hand, an enrichment culture containing a type II methanotroph was isolated from soils that oxidize atmospheric methane and then was experimentally adapted to nanomolar values of K_m close to those found in aerobic soils [20]. The enrichment methane-oxidizing cultures that we isolated from samples of forest soil on methane-rich media (10–20% in the gas phase) contained bacteria of the genus *Methylocystis*. In preliminary experiments, these associations were shown to be able to oxidize methane at atmospheric concentrations (Bykova, unpublished data). The obtained data support the hypothesis that methanotrophs with low affinity to methane can adapt to its oxidation at atmospheric concentrations. This hypothesis has now been tested in our experiments involving pure and mixed cultures of methanotrophs including collection strains and isolates from forest soil.



Kinetics of atmospheric methane uptake in samples of gray forest soil obtained from (*1*) "Agrocenosis" test plot and "Forest" test plot (*2*) without acetylene and (*3*) with 5% acetylene introduced in the gas phase.

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