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ECOLOGY

Anaerobic Methane Oxidation in Soils and Water Ecosystems

L. A. Pozdnyakov*, A. L. Stepanov, and N. A. Manucharova*****

Soil Science Faculty of Moscow State University, Moscow, Russia

**e-mail: APL-223@mail.ru;*

***e-mail: Stepanov_aleksey@mail.ru;*

****e-mail: manucharova@mail.ru*

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Abstract—The process of anaerobic methane oxidation has been studied for over 30 years on the example of bottom marine sediments and fresh water ecosystems. This paper presents a review of the results of these investigations. It is also demonstrated that this process can proceed not only in submerged but also in drained peat and automorphic sod-podzol soils. The latter soils continue to absorb methane after anaerobic conditions are created and an inhibitor of methane monooxygenase (acetylene) is introduced. Oxidated compounds (nitrates, sulphates) are stimulatory to gas absorption; deoxidated nitrogen compounds (ammonium chloride) do not produce significant changes. Incubation with the addition of nitrates and sulphates in an atmosphere of argon and methane results in an increase in the fraction and abundance of archaea in the soil. This is in agreement with the data obtained for aquatic inhabits.

Keywords: methane, anaerobic methane oxidation, denitrification, peat soils

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INTRODUCTION

The process of anaerobic methane oxidation was discovered in bottom marine sediments about 30 years ago and, since then, has mainly been studied on the example of aquatic habitats. At present, a quantitative assessment of the role played by anaerobic methane oxidation in the global cycle of this greenhouse gas has been performed for the world ocean. Total numbers are estimated to be between 75 and 300 Tg of CH_4 per year [20]. Not only do these values exceed by many times the rate of the total methane flux from the ocean to the atmosphere (about 10–15 Tg per year [22]), but they are also comparable with total methane emission (about 500–600 Tg per year [22]).

The experimental data have been inadequately collected, so the mentioned numbers should be viewed carefully. However, the order of magnitude itself testifies to the importance of the process on a global scale and accounts for the relevance of studies in this field. Nonetheless, attempts to evaluate the significance of anaerobic oxidation in terrestrial ecosystems have not yet been made. When examining the methane balance in soils, it is still supposed as before that only methanogenic activity leading to the release of methane takes place under anaerobic conditions, while the methanotrophic activity consists in its absorption under aerobic conditions.

The first works devoted to anaerobic methane oxidation showed that this process has a fully biological nature [39].Research on anaerobic methane oxidation is complicated by the fact that methane-oxidizing organisms have not been isolated into pure or binary cultures. The development and observation of enrichment cultures has proven effective [1, 2, 17, 32, 34]. In addition, the possibility of methane oxidation under anaerobic conditions was shown for some cultivated species of methanogens (*Methanosarcina barkeri*, *M. acetivorans*, *Methanobacterium thermoautotrophicum*, etc.) [28, 39]. However, the low rates of the process (two to three orders of magnitude lower than the rate of formation of CH_4 by the same culture) make it impossible to attribute the scale of anaerobic oxidation observed in nature to these organisms. The possibility of co-oxidation of trace methane by pure cultures of sulphate-reducers has also been reported [19].

By now two types of the anaerobic methane oxidation coupled to sulphate-reduction and denitrification are reliably known. The first process is better understood. It is considered to be performed by a consortium of non-culturable archaea of ANME-1, ANME-2 and ANME-3 related to the different orders of methanogens, mainly Methanosarcinales and sulphate-reducing eubacteria related to *Desulfosarcina*, *Desulfococcus* and *Desulfolobus* species [26]. The functioning of the consortium is only possible under strictly anaerobic conditions. The methane oxidation is not only an energy source but also a biomass source for these organisms. Due to inclusion of ^{13}C-depleted methane at cold seeps in composition of nucleic acids and lipids, the classification position of methane-oxidizing anaerobes has been established [21].

Later, specific probes were developed and the existence of a methane-oxidizing consortium was proved by FISH method [14, 33]. The biochemistry of this process is not clearly understood. Despite active research [27, 32, 37], it remains to see how metabolism of the consortium components is related and what material is an interspecific intermediate. Hydrogen, acetate, methanol, formaldehyde and etc. are proposed for it, but convincing arguments for one of them are not still given. The possibility of the existence of organisms which are capable of performing the whole process and do not need to aggregate with others is not ruled out. In this connection, the information on revealing free-living or microcolony-forming cells, mainly the ANME-2, and also ANME-1 and ANME-3 is of interest [15, 26, 33].

The use of nitrates and nitrites for anaerobic methane oxidation was clearly demonstrated only several years ago [34]. Initially, it was postulated that the process was performed by a consortium of ANME and non-culturable denitrifying eubacteria in this case. However, further studies showed evidence that open eubacteria could oxidize methane under anaerobic condition independently [17].

Works in the literature devoted to anaerobic methane oxidation in natural and technogenic habitats that are different from the bottom sediments are not numerous, although they demonstrate its widespread occurrence. The behavior of the process has been shown in the water column of fresh lakes where the archaea cells of ANME-1 and ANME-2 were found [15]. Hydro-chemical evidence for anaerobic methane oxidation has been obtained for the underground waters near landfills [16]. Methane oxidation in the rumen has been reported [23]. The behavior of the process has been fixed in rice soils [29, 30, 31]. The stimulating effect of SO_4^{2-} and NO_3^- on its rate has been shown. The obtained data are contradictory regarding Fe^{3+} and MnO_2 [25, 31]. Later, anaerobic methane oxidation was demonstrated in peat bog soils [36]. The authors attribute this mainly to NO_3^-, although the involvement of other compounds, including organic ones, has not been ruled out. In line with the above, our objective was to establish the fact of anaerobic methane oxidation in unsubmerged soils not touched on by researchers of this process, as well as to provide an initial estimate of its rate.

OBJECTS AND METHODS

Research was conducted in the cultivated lowland peat soils (classification of 1977) of the floodplain of the Yakhroma River (Dmitrov raion, Moscow oblast). Peat formation began here during the preglacial period before the present riverbed of the Yakhroma was formed and its alluvial deposits were generated. The peat is of lowland and partly intermediate type; woody, grass-woody, sedge, hypnum-sedge, and hypnum types [4, 9]. Its thickness carries lenses of alluvium and lake deposits.

We investigated the Blizhnii agricultural grounds of the Dmitrov branch of the All-Russia Research Institute of Reclaimed Land Agriculturaltural Management, which was drained in 1914. The present groundwater level is maintained, on the average, at a depth of 1–1.5 m in the course of the year [9].

The general formula for the soil profile is as follows: Aarab (0–30 cm) is heavily decomposed peat, with no traces of peat-forming vegetation, brown in color, with weak cloddy-blocky structure; Tinter (30–50 cm) is heavily decomposed peat with plant residues in minor amounts; T1 (50–90 cm) is somewhat decomposed peat; T2 (below 90 cm) is the least decomposed peat, reddish in color, quickly darkening in air, and composed of coarse wood pulp.

In soil composition and characteristics, the agricultural grounds are well divided into near-bed, central, and near-terrace areas. Prior to drainage of the bog, a spring bog formed in the near-terrace part of floodplain, where substances (carbonate and iron compounds) derived from a watershed accumulated; even today there is a periodic discharge of ground water. The surface soil layer of this part of the floodplain is high-ash (45–85%) and has low acidity; pH_{salt} can reach 7.7. The other area of mineral substance accumulation is formed in the near-bed part, where ash content reaches the same values. The central floodplain part, on the contrary, is characterized by low ash content (10–30%).

Samples were taken by a drill from the arable layer with a depth of 10–30 cm. The geographic coordinates were determined with a Garmin GPS-72 receiver. The distribution of values was mapped with Surfer 8.0 program using the Kriging interpolation method.

Considering the fact that anaerobic methane oxidation has been unstudied in soils dominated by oxidizing conditions, we explored sod medium-podzolic cultivated soil (classification of 1977) uncovered under a deposit on the territory of the Chashnikovo experimental soil ecology training center of Moscow State University. One sample of the arable layer was taken.

Measurements of the response of methane absorption to the addition of an electron acceptor to the soil were carried out. The results were similar to the measurements for submerged soils [25, 31]. KNO_3 and Na_2SO_4, for which the possibility of the methane oxidation has been proven, were used. Three-gram soil samples were incubated in 15 ml serum vials. The quantity of the added KNO_3 amounted to 0.4 mg/g, as in the standardized denitrification determination technique using the acetylene method [5, 10]. The salt cake was added in an equivalent quantity of 0.3 mg/g under the assumption that it would be reduced to sulphide. This is typical of the anaerobic methanotroph-

Description of the used nucleotide probes

Zone	Target group of organisms	Target plot 16S rRNA	Nucleotide sequence of the prob (5'–3')	Formamide, %*	NaCL, mM**	Reference
EUB338 I	Bacteria	338–355	GCT GCC TCC CGT AGG AGT	20	225	[13]
EUB338 II	Bacteria (Planctomycetales)		GCA GCC ACC CGT AGG TGT			
EUB338 III	Bacteria (Verrucomicrobiales)		GCT GCC ACC CGT AGG TGT			
ARCH915	Archaea	915–934	GTG CTC CCC CGC CAA TTC CT	30	112	[35, 38]
ARC344		344–363	TCG CGC CTG CTG CIC CCC GT			

* Formamide concentration in the transfer buffer.

** NaCL concentration in the washing buffer.

related organisms [6, 26]. Ammonium chloride (0.21 mg/g) and glucose (2.5 mg/g) were also added. The soil was saturated with water to its maximum water holding capacity; the vial was purged with argon for one minute. Next we added 1 ml of acetylene—an MMO inhibitor [3] that blocks N_2O reduction [5, 10]. The methane concentration produced in the gas phase of the vial amounted to 600–900 ppm. The initial methane content was measured one day after the substrates were added. The final content was measured after four days of incubation at room temperature.

The response of denitrification, estimated by the usual acetylene method [5,10], to the addition of a large amount of methane (up to 10%) in the gas phase of the vial was measured. The incubation period made up a day in this case.

The methane concentration was measured by a Chrom-41 chromatograph equipped with a flame ionization detector. Spherosil was used as a column filler and argon served as a carrier gas. Nitrous oxide was determined with a chromatograph (model 3700/4, Chromatograph plant) equipped with a thermal conductivity detector (a catarometer) on a Polysorb-1 column. Helium served as a carrier gas. The oxygen content was measured with a Kristall 5000.1 chromatograph equipped with a thermal conductivity detector. In all cases the sample volume amounted to 0.5 cm^3.

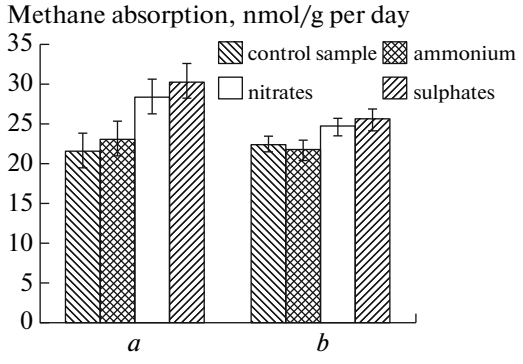

Fig. 1. Methane absorption in peat soil under anaerobic conditions: (*a*) without addition of glucose and (*b*) with addition of glucose.

The KNO_3 and Na_2SO_4 sod-podzolic soil samples incubated using the procedure described above, as well as a control sample undergoing only moistening before incubation, were studied by the FISH method. A suite of rRNA-specific oligonucleotide probes which have been developed to detect representatives of the domains Bacteria and Archaea was applied for hybridization (table). Synthesis of the probes, which were stained with 6-diamidino-2-phenylindole (DAPI), was performed by the Synthol company (Moscow, Russia).

In order to separate cells from soil particles, 1 g of soil was dissolved in 10 ml of sterile water and was sonicated for 2 min at a frequency of 22 kHz. The suspension was centrifuged several times for 10 min to precipitate large particles: first three times at 1000 rpm (removing the precipitation), then at 10000 rpm. The resulting precipitation was resuspended with sterile distilled water to a volume of 2 ml. The cells were fixed using formaldehyde, for which 0.4 g of paraformaldehyde was dissolved in 10 ml of phosphate buffer (pH 7) in a water bath. The cells, which were precipitated for 5 minutes at 10000 rpm, were resuspended in 0.5 ml of phosphate buffer (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g NaH_2PO_4, 1 l H_2O, pH 7.0) with the addition of 1.5 ml of 4% formaldehyde solution in phosphate buffer. They were then incubated for 1.5 h in a shake-flask propagator at room temperature.

The fixed cells were precipitated at 8000 rpm for 2 min, washed twice with phosphate buffer, and resuspended in a mixture of ethanol and phosphate buffer (1 : 1). Before the procedure, the samples were kept at 20°C. One milliliter of the cell suspension of the fixed sample was spread on gelatin-coated slides with windows separated by a Teflon coating. The fixed cell samples spread on the slides were treated with a lysozyme solution (10 mg in 1 ml 0.05 M EDTA and 0.1 M TRIS HCI, 1 : 1, pH 8.0) to increase the cell

wall permeability of the bacteria. The samples were stored at room temperature for one day. The slides were then washed in a successive series of ethanol solutions (50, 80, and 96%) and dehydrated. The hybridization of the samples with fluorescently labeled probes was performed using a standard procedure [38] at 46°C. The hybridization conditions used for different probes are given in the table.

The samples were analyzed with a Zeiss Mikroskop Axioskop 2 plus luminescence microscope. The magnitude of the target micropopulation in the samples was determined by counting the number of hybridizated cells using probes in 80 fields of the microscope in one cell, with a subsequent calculation per 1 g of soil.

RESULTS AND DISCUSSION

The effect of the addition of electron acceptors on anaerobic methane oxidation was studied on the example of a soil sample from the central part of the floodplain. Methane oxidation in the control sample took place at a rate of 22 nmol/g per day. The addition of nitrates and sulphates resulted in a one-third increase in methane absorption over the control (Fig. 1).

The following explanations for the given growth can be suggested: stimulation of the anaerobic methanotrophs by increased availability of the electron acceptors; stimulation of the methanotrophs by improvement of mineral nutrition conditions; enhancement of methane oxidation under the growth of anaerobic bacteria on the other substrates; activity of the aerobic methanotrophs using the residual oxygen in the vials.

The addition of acetylene as a suicide inhibitor of both forms of the key enzyme of the aerobic methanotrophy—methane monooxygenase in the vials [3] allows a complete neutralization of the aerobic methanotrophs to be expected.

The aerobic methanotrophs are suggested to use another enzyme system, which is not susceptible to acetylene. The methanogenic enzymes catalyzing the backward reactions play the most important role, which is supported by the fact that the process is inhibited by 2-bromoethanesulfonic acid and sodium molybdate (inhibitors of methanogenesis and sulphate-reduction, respectively) [39]; in addition, methyl-CoM-reductase genes and others were found in ANME-clusters of the archaea [18]. This is shown for anaerobic methane oxidation associated with sulphate-reduction. If nitrogen compounds are used, another mechanism occurs. This mechanism is still unknown and excludes the participation not only of methane monooxygenase but of methyl-CoM-reductase as well [17].

Measurements showed that, with our procedure, the residual concentration of O_2 in the gas phase of the vial made up 0.05–0.40%. An oxygen-content dependence of the obtained data was not found. The indicated values are not barriers to the development of anaerobic bacteria in soil where anaerobic microzones are present, even when the samples are incubated in atmospheric air [11, 12]. On the contrary, the development of aerobic methanotrophs in these conditions is hindered. According to literature data, the optimal oxygen content is 15–17% for some species and 30–45% for others; when the content is less than 1%, oxygen-limited growth of *Methylococcus capsulatus* is shown [2].

The fact that nitrates act precisely as the electron acceptor in our case is supported by the amplification of denitrification from 50.0 to 68.6 nmol N_2O/g per day for addition to the gas phase of the methane vial. The control variant with the addition of reduced nitrogen compound (NH_4Cl) did not demonstrate either an apparent methane increase or an inhibition of the methane absorption. Both the nitrates and ammonium salts are available sources of nitrogen for a great number of aerobic methanotrophs, and in our experiment the concentration of NH_4Cl did not reach levels at which this and other ammonium compounds interfere with the development of aerobic methanotrophs [2, 24]. In addition, when aerobic methane absorption was measured (without displacement of air by argon or the addition of acetylene), we did not observe a stimulation of this process due to the addition of nitrates. The addition of glucose in the control sample and the sample with ammonium chloride did not cause changes in the intensity of methane absorption (Fig. 1), but the increase in methane absorption in the samples with the addition of KNO_3 and Na_2SO_4 proved to be distinctly smaller than without glucose.

It can be concluded that the methane absorption in our case is performed by specific anaerobic autotrophs, apparently competing with heterotrophs for electron acceptors.

After the possibility in principle of anaerobic methane oxidation in drained peat soils was recognized, an estimation of the spatial distribution of potential process activity was made on the Blizhnii plot. Two indicators were used, such as the total absorption of methane in the control sample and the response of the soil's microbial community to the addition of nitrates as a more significant electron acceptor in peat soils.

Methane absorption in the control sample ranged from 6.8 to 35.7 nmol/g per day (an average of 18.7); the response in all samples was positive and amounted to 0.3–8.6 nmol/g per day (an average of 3.8). The increase of methane absorption was from 2.3 to 65.7% (an average of 26%) relative to the control sample, and no dependence on the control values for the same sample was found. These values in the drained peat soils proved to be several orders of magnitude lower than the data obtained by S. Kumaraswamy et al. [25] using a similar procedure for submerged rice soils: 40 μmol/g per day in the control sample and

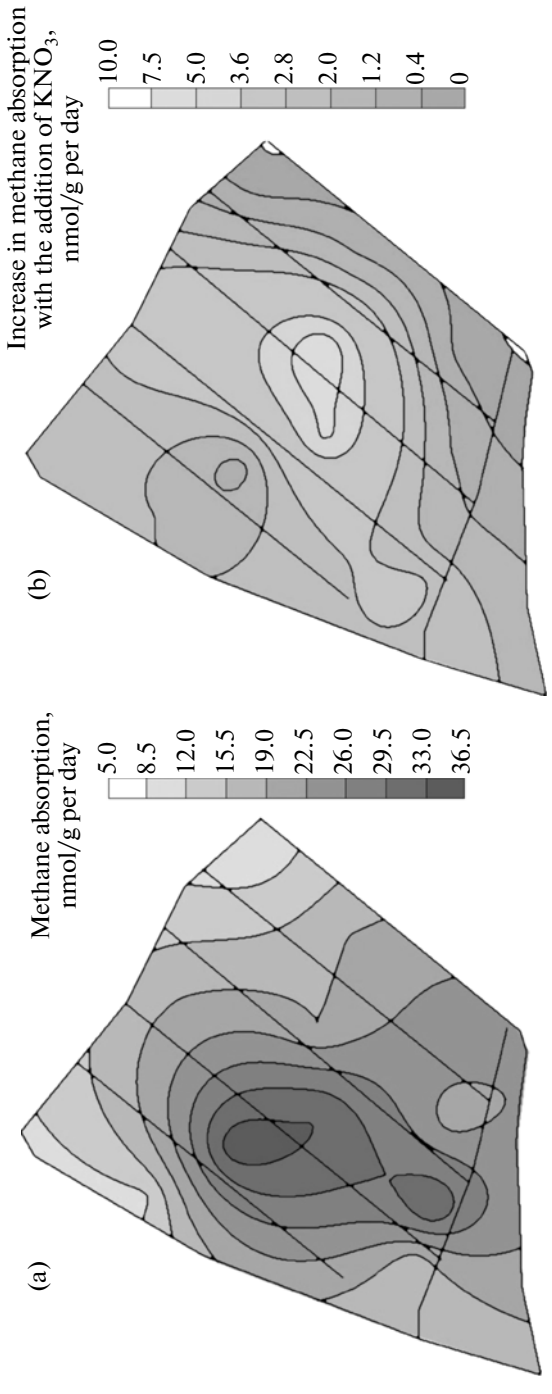

Fig. 2. Distribution in the topsoil of the Blizhnii plot: (a) values of methane absorption in the control sample and (b) increase in methane absorption with the addition of KNO_3.

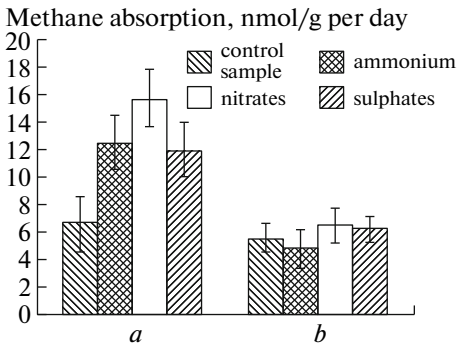

Fig. 3. Methane absorption in soddy-podzolic soil under anaerobic conditions: (*a*) without addition of glucose and (*b*) with addition of glucose.

170 μmol/g per day with the addition of KNO_3. According to the data by K.A. Smemo et al. [36], the average rate of anaerobic methane oxidation for certain undrained peat soils was 17 nmol/kg per second, corresponding to 1.5 μmol/g per day.

The potential methane formation activity which we measured previously [8] depended on the location in the sections which were perpendicular to the bed of the Yakhroma and differed from the method of agricultural use. For this reason a distribution over sections was formed for the total nitrogen content [7]. At the same time, the relation to the location in all areas of the floodplain (near-bed, central, and near-terrace) was found for the potential level of soil respiration.

The distribution of methane absorption in the control sample and its increase with the addition of nitrates proved to be ambiguous (Fig. 2). Moreover,

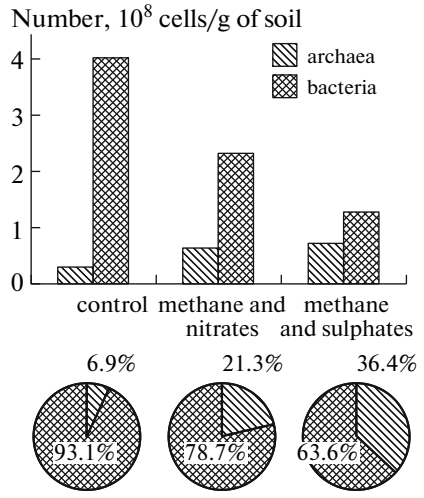

Fig. 4. Change of the number (above) and ratio (below) in the soil prokaryotic community of bacteria and archaea with the addition of anaerobic methane oxidation substrates.

the increase in methane absorption reaches the highest levels in sections II and III (the numbering is from southeast to northwest), where methane formation is less active; the lowest levels are in sections I and IV, where it is maximal. It is not inconceivable that the activity of the anaerobic methanotrophs also made a contribution to the observed variation in the soil methane emission. The role of the anaerobic oxidation of methane in the balance of this greenhouse gas in surface biogeocenosis has yet to be determined.

Taking into consideration the lack of works devoted to the anaerobic oxidation of methane in automorphic soils, we studied a sample of soddy-podzolic soil of the watershed in the same edaphic-climatic zone. The increase in methane absorption under anaerobic conditions with the addition of nitrates and sulphates leveing out under the effect of glucose (Fig. 3), as well as the amplification of denitrification from 62 to 74 nmol of N_2O/g per day with the addition of methane in the gas phase, were characteristic of this sample. The main differences can be summarized as follows: first, a low absorption of methane in the control sample compared with the values observed in the soils of the near-bed areas of the Yakhroma floodplain, which were enriched in mineral sediments; second, a relative increase in methane absorption with the addition of nitrates exceeding all similar values calculated for peat soils, reaching 2.6 times. The absolute increase in this case corresponded to the average values for the peat soils. From the above it is clear that microorganisms conducting the process are present in soddy-podzolic soils, functioning in the anaerobic zones of the aggregates and oxidizing biogenic methane. However, methane here is a less significant substrate for anaerobic bacteria than in the drained peat soils; a smaller number of organisms conduct anaerobic oxidation under ordinary conditions, while their activity increases greatly when the nitrogen limitation is eliminated. In this connection, more abrupt changes in the structure of the microbial community in the soddy-podzolic soils than in the peat soils are likely if they are placed in favorable conditions for the anaerobic oxidation of methane. The differences of the community formed following a succession caused in this way will be determined by the development of anaerobic methanotrophs. We activated this succession and studied it by the FISH method.

In samples incubated for 4 days in an atmosphere of argon and methane with the addition of KNO_3 and Na_2SO_4, we observed a reduction in the absolute number of countable cells of bacteria by 1.8–3.1 times compared with the control sample and a simultaneous increase in the number of archaea by 2.1–2.5 times (Fig. 4). This caused a change in structure of the prokaryotic community of the soil. If the ratio of archaea was not in excess of 7% in the control sample, it reached 20% in the sample with nitrates and more

than 35% in the sample with sulphates. These data agree with the concepts of the structure of the microbial complex of the anaerobic methane oxidation which formed in the studies of bed sediments. An average of 100 cells of the archaea of the ANME clusters and 200 eubacteria-sulphate-reducers are part of the group producing methane oxidation by means of sulphates [14]. When nitrates and nitrites were used as the electron acceptor, the ratio of eubacteria and archaea proved to be wider and was equal to 8 : 1 [34]; in a prolonged period of cultivation, archaea were completely eliminated [17]. In our research, a distinctly high population of eubacteria survived in the sample with nitrates compared with the sulphates.

CONCLUSIONS

The possibility of the anaerobic oxidation of methane using both sulphates and nitrates in unsubmerged soils with predominant oxidizing conditions, including automorphic, was shown for the first time. The process can take place within the anaerobic microzone of the aggregates in these soils and contributes to the taking up of the methane emitted by the soil at an earlier stage, before falling within the zone of activity of the aerobic bacteria. The coupling of the anaerobic oxidation of the methane and methane formation can be rather close. The absolute increase in methane absorption under anaerobic conditions with the addition of nitrates (used as an oxidizer) reached the highest levels in the same areas of the floodplain where the potential activity of methane formation was minimal, and vice versa. When favorable conditions for the development of anaerobic methanotrophs are created, the archaea grow intensively, especially in the case of methane oxidation using sulphates.

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