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Article

Aerobic Methanotrophs in Natural and Agricultural Soils of European Russia

Anna Kizilova [1,*], Andrey Yurkov [2] and Irina Kravchenko [1]

[1] Winogradsky Institute of Microbiology, Russian Academy of Sciences, Prospekt 60-letiya Oktyabrya, 7/2, Moscow 117312, Russia; E-Mail: irinakravchenko@inbox.ru

[2] Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstrasse 7B, Braunschweig 38124, Germany; E-Mail: andrey.yurkov@dsmz.de

* Author to whom correspondence should be addressed; E-Mail: alegrria@gmail.com; Tel.: +7-499-135-7573; Fax: +7-499-135-6530.

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Abstract: Human activities such as land management and global warming have great impact on the environment. Among changes associated with the global warming, rising methane emission is a serious concern. Therefore, we assessed methane oxidation activity and diversity of aerobic methanotrophic bacteria in eight soil types (both unmanaged and agricultural) distributed across the European part of Russia. Using a culture-independent approach targeting *pmoA* gene, we provide the first baseline data on the diversity of methanotrophs inhabiting most typical soil types. The analysis of *pmoA* clone libraries showed that methanotrophic populations in unmanaged soils are less diverse than in agricultural areas. These clone sequences were placed in three groups of, so far, uncultured methanotrophs: USC-gamma, cluster I, and pmoA/amoA cluster, which are believed to be responsible for atmospheric methane oxidation in upland soils. Agricultural soils harbored methanotrophs related to genera *Methylosinus*, *Methylocystis*, *Methylomicrobium*, *Methylobacter*, and *Methylocaldum*. Despite higher numbers of detected molecular operational taxonomic units (MOTUs), managed soils showed decreased methane oxidation rates as observed in both *in situ* and laboratory experiments. Our results also suggest that soil restoration may have a positive effect on methane consumption by terrestrial ecosystems.

Keywords: methane oxidation; soil; land management; agriculture; methane monooxygenase; aerobic methanotrophs

1. Introduction

The European part of Russia spans over about four million square kilometers and includes a wide diversity of both cultivated and natural landscapes. Human activity associated with the population growth promoted the conversion of natural ecosystems into agricultural areas, resulting in deep alterations of entire ecosystems, including the belowground areas [1]. Changes in vegetation, soil properties, and nutrition greatly affect microbial population [1–4] facilitating further modifications of biogeochemical cycles of main biogenic elements such as carbon, nitrogen, and phosphorous.

Being an essential part of the carbon turnover, methane is also commonly recognized as an important agent of the greenhouse effect [5] as it captures 20–30 times more infrared radiation than carbon dioxide. In terrestrial ecosystems, methane is produced by methanogenic Archaea and via geological processes. Methane is mainly oxidized photochemically in the stratosphere, however some terrestrial environments, such as soils, may also consume this greenhouse gas, contributing up to 6% of the global methane sink [6]. In particular, aerobic bacteria, utilizing methane as the source of carbon and energy (methanotrophs) are described in phyla *Alpha-* and *Gammaproteobacteria* [7], the latter including filamentous bacteria *Crenothrix* and *Clonothrix* [8,9]. In addition, the ability to oxidize methane aerobically was demonstrated for the extremely acidophilic methane-oxidizing Verrucomicrobium [10,11]. Thus, soil microbiota may accomplish two distinct functions, emission of methane by methanogens and also methane consumption by diverse methanotroph population. Being a significant component of the natural carbon cycle, microbial methane metabolism is ultimately affected by land management, including deforestation, grazing, and application of fertilizers. However, still little is known about the responses of methanotrophic bacteria to land-use.

In bacterial cells, methane conversion to methanol is performed by methane monooxygenase enzymes, soluble methane monooxygenase (sMMO), and particulate methane monooxygenase (pMMO). The gene *pmoA* is encoding the 27 kDa subunit of pMMO and is present in all currently known methanotrophs, except *Methylocella* [12] and *Methyloferula* [13]. Therefore, this gene has been successfully used to detect and identify methanotrophs in various environmental samples [14,15]. Furthermore, good correlation between phylogenies inferred using *pmoA* and 16S rRNA genes has been demonstrated e.g., [16]. It is worth mentioning that most of currently known cultured methanotrophs are not able to maintain their growth under low methane concentration for prolonged times [17], except for a *Methylocystis* strain, SC2, possessing an izozyme of pMMO with high affinity to methane [18]. Therefore, proper culture-dependent biodiversity assessment of methanotrophs is difficult and, therefore, may be strongly biased. Culture-independent techniques targeting *pmoA* gene provide certain advantages and may deliver essential information about the structure of the methane-oxidizing community, including those organisms, which still remain uncultured.

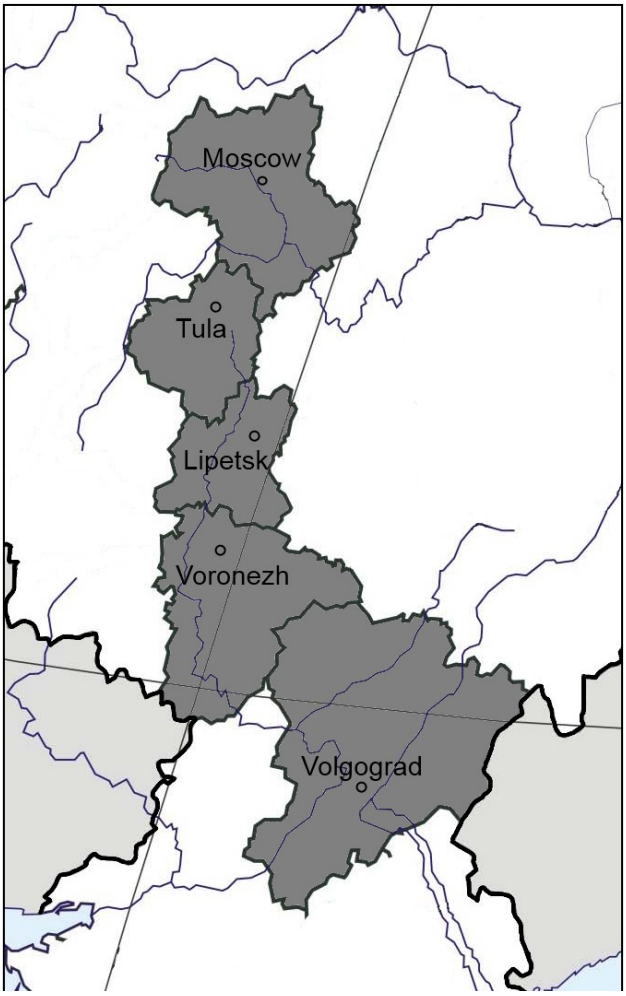
This paper reports results of the study aimed at the estimation of effects of land management on methanotrophic bacteria in five zonal soils, distributed across the European part of Russia. We also analyzed three intrazonal soils, which are not subject to agriculture. We hypothesized that land management substantially affected activity and diversity of methane oxidizing bacteria in soils. We analyzed methane oxidation rates in field and laboratory conditions, and also assessed diversity of methanotrophic bacteria using a culture-independent approach consisting of amplification and cloning of *pmoA* gene fragments.

2. Experimental Section

2.1. Soil Sampling Sites

Soil samples were collected from five zonal and three intrazonal soil types during an expedition in 2008 between 20 June and 15 July (Figure 1). For each zonal type of soil (Phaeozems excluded), natural and agricultural variants were sampled. Intrazonal soils are not subject to agriculture, which is why only unmanaged variants were studied. Additional information on soil sampling sites is provided in Table 1.

Figure 1. Map of the European part of Russia, where the soils samples were collected.



After gas phase sampling, soil blocks were cut out from the 0–10 cm soil layer from under the chambers. Litter was excluded from analysis. These soil blocks were used to determine basic soil properties (e.g., organic matter and nitrogen content, pH, *etc*.) and to study methanotrophic bacteria and potential methane oxidation. Soil samples with natural moisture content were mixed, sieved (2 mm), and stored at 8–10 °C in aerated plastic bags prior to analyses. All analyses and DNA extraction were performed immediately upon arrival. Basic properties of these soils and surface fluxes of methane are also provided in Table 1.

Table 1. Description of soil sampling sites and selected soil parameters.

Region, site (coordinates)	Soil type, FAO	Land management	Vegetation	C org, (%)	N tot, (%)	C:N	pH (KCl)	NO_3+NH_4, mg/100 g	Surface methane flux (μg CH_4 m^{-2} h^{-1})
Moscow, Puschino (54.50°N, 37.37°E)	Podzoluvisol	unmanaged	Mixed forest (*Pinus spp.*, *Abies spp.*, *Betula spp.*)	2.1	0.2	10.5	4.6	0.8	−19.0 ± 3.4
		managed	Barley field (*Hordeum vulgare*)	1.2	0.1	11.9	5.3	0.8	−2.6 ± 1.2
Tula, Schekino (54.00°N, 37.31°E)	Luvisol	unmanaged	Broadleaf forest (*Tilia sp.*, *Corylus spp.*, *Ulmus spp.*)	1.3	0.1	10.5	4.2	2.3	−26.0 ± 8.1
		managed	Wheat field (*Triticum spp.*)	0.9	0.1	9.2	5.1	0.8	−4.3 ± 1.1
Lipetsk, Danki (53.30°N, 38.58°E)	Phaeozem	unmanaged	Broadleaf forest (*Acer spp.*, *Quercus spp.*, *Populus spp.*, *Tilia spp.*)	4.3	0.3	12.5	5.8	2.6	−19.0 ± 2.0
Voronezh, Bobrov (51.07°N, 40.17°E)	Solodic chernozem	unmanaged	Birch and aspen forest (*Betula spp.*, *Populus spp.*)	2.7	0.2	10.8	6.4	0.9	+6.1 ± 0.4
Voronezh, Talovaya (51.07°N, 40.43°E)	Solonetz	unmanaged	Grassland (*Bromopsis spp.*, *Vicia spp.*, *Alopecurus spp.*, *Phleum spp.*)	2.9	0.2	11.7	8.2	1.2	+12.9 ± 2.0
Voronezh, Talovaya (51.07°N, 40.43°E)	Chernozem	unmanaged	Grassland (*Stipa spp.*)	4.3	0.4	11.5	6.7	2.0	−7.6 ± 2.6
		managed	Wheat field (*Triticum spp.*)	3.9	0.3	11.3	6.2	2.0	−2.2 ± 1.2
Volgograd, Kachalino (49.49°N, 44.32°E)	Gleyic kastanozem	unmanaged	Elm and alder forest (*Ulmus spp.*, *Alnus spp.*)	3.2	0.3	11.9	6.9	0.8	−24.0 ± 7.0
Volgograd, Ylovlya (49.47°N, 44.31°E)	Kastanozem	unmanaged	Grassland (*Festuca valesiaca*, *Euphorbia spp.*, *Artemisia spp.*)	1.6	0.1	10.9	5.5	0.7	−30.0 ± 5.2
		managed	Wheat field (*Triticum spp.*)	0.9	0.1	9.1	5.8	0.6	−25.0 ± 7.0

2.2. Surface Methane Flux Measurements

Methane fluxes have been measured by means of the static chamber method [19]. Therefore, 15-cm-high organic glass chambers with a hydraulic valve and a steel 32 × 32 cm bottom, cut to a depth of 20 cm, were placed over intact soils. A total of three replicates were made for each soil. The gas phase was sampled with a syringe and transferred into 9 ml pre-evacuated tubes (Vacuette, Grenier Bio-one, Austria) for further measurements by gas chromatography in the laboratory.

2.3. Potential Methane Oxidation Rate ($^{14}CH_4$)

Methane oxidation rates in soil samples were measured by means of radioisotope tracer technique utilizing ^{14}C-labeled methane. Water solution of $^{14}CH_4$ (0.08 MBq; Izotop, Russia) was added with a syringe to 20 mL glass tubes with soil samples (5 g), which were sealed hermetically with gas-tight rubber caps. Final concentration of radioactively-labeled methane was approximately equal to 10 nL mL^{-1} (1.3 nmol CH_4 g^{-1}) or 10 ppm. Samples of soils were then incubated at room temperature for 72 h. After incubation, the samples were fixed with 2 mL of 1 N KOH to stop microbial activity and processed according a previously described protocol [20]. Soil samples with an added 1 N KOH solution, before adding radioactively-labeled methane, were used as controls. All measurements were done in triplicate.

2.4. DNA Extraction and PCR Amplification

DNA was extracted from 0.25 g of a mixed sieved (2 mm) soil sample (the upper 0–10 cm layer) with PowerSoil DNA Kit (MO BIO Laboratories, Carlsbad, USA) according manufacturer's recommendations. DNA extraction was performed in triplicates. Thereafter, DNA probes representing the same soils were pooled and used for PCR amplification. PCR reactions were performed in triplicates and amplified products were pooled together for the further analysis. Soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO) in investigated samples was targeted with primer pairs mmoX206f and mmoX886r [21], and A189 and A682 [22], respectively. To amplify both gene regions we used a touchdown PCR consisting of following steps: three minutes of initial denaturation at 94 °C followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s (with 0.5 °C temperature drop at each cycle), and extension at 72 °C for 45 s. A final extension step of 5 min at 72 °C was conducted. Amplification was performed on MyCycler thermocycler (BioRad Laboratories, Hercules, CA, USA). Amplified PCR-products were purified with QIAquick Gel Extraction Kit (Qiagen, Germany).

2.5. Construction of Clone Libraries

Purified PCR products were cloned into the pGEM-T vector using Easy Vector System I (Promega Corporation, Fitchburg, WI, USA) and competent cells of *E. coli* DH10B strain. Transformed cells were spread over solid LB medium, supplemented with ampicillin and X-gal, and grown overnight at 37 °C. A total of one hundred positive (white) clones were selected from each library (a total of 12 libraries have been constructed), and a colony-based PCR was performed with universal plasmid primers M13F and M13R. The presence of desired insert was checked with agarose (1.2%) electrophoresis.

2.6. DNA Sequencing and Phylogenetic Analysis

Clones showing the insert were sequenced commercially (Syntol, Russia). Primer and vector sequences were deleted from sequences. Nucleotide sequences were compared with sequences available in the NCBI GenBank database using the BLASTn online tool [23]. At this step, molecular operational taxonomic units (MOTUs), consisting of fully identical nucleotide sequences were defined. A total of 22 MOTUs, from a total of 12 *pmoA* clone libraries, were deposited in GenBank under accession numbers JF780897-JF780911 and KC923237-KC923243. Similarity values given in text refer to amino acid sequences. For phylogenetic analyses, nucleotide sequences were translated into amino acid sequences and aligned using BioEdit software [24]. Maximum likelihood phylogenetic analysis was performed from aligned amino acid sequences (156–160 amino acids) with RaxML algorithm (version 7.3.0) implemented in raxmlGUI [25] and the BLOSUM62 substitution model in the PROTCAT option, followed by 100 rounds of bootstrap replicates [26].

Species accumulation curves were calculated with EstimateS 8.2 using 50 randomizations, sampling without replacement, and default settings for upper incidence limit for infrequent species [27]. Four estimators of species richness were used: Chao 2 richness estimator, ICE incidence-based coverage estimator, Jackknife 1 first-order Jackknife richness estimator, and Bootstrap richness estimator. Of the four species richness estimators, ICE distinguishes between frequent and infrequent species in analyses, Bootstrap does not differentiate the species frequency, and the first-order Jackknife richness estimator additionally relies on the number of species only found once. Chao 2 estimator is distinct from the other species estimators as it is an incidence-based estimator of species richness, which relies on the number of unique units and duplicates (species found in only one and two sample units). Other details on the use of species richness estimators are given by Yurkov *et al.* [28].

3. Results and Discussion

3.1. CH_4 Surface Flux and Potential Methane Oxidation Rate

This study was originally aimed at providing first baseline data on methane metabolism in studied soils, and detailed studying of ^{14}C balance was not in the scope of our survey. Mostly due to the lack of available reference data on analyzed soils, this study was designed to reveal methane metabolism driven by soil microbes. Therefore, we have assessed complete oxidation of methane (at near atmospheric concentrations) to carbon dioxide, methane incorporation into microbial biomass (including metabolites and dissolved organic matter), and potential methane oxidation rate. We did not measure distribution of ^{14}C in various types of metabolites, different biomass components, and did not estimate remaining pools of ^{14}C after the incubation. Thus, we do not provide detailed information regarding ^{14}C balance in studied soils.

All unmanaged soils, with the exceptions of Solodic Chernozem and Solonetz, showed negative surface methane fluxes, being, thus, sinks of atmospheric methane (Table 1). Maximum surface methane oxidation values in natural soils were recorded for Podzoluvisol and Kastanozem being 19 and 30 μg CH_4 m^{-2} h^{-1}, respectively. Solodic Chernozem emitted up to 6 μg CH_4 m^{-2} h^{-1}. Solonetz showed positive methane flux reaching 13 μg CH_4 m^{-2} h^{-1}. In contrast, natural Chernozem demonstrated negative surface methane flux being −7.6 μg CH_4 m^{-2} h^{-1}. Although methane

consumption by tundra and forests soils has been observed previously [29,30], our results suggest that unmanaged steppe biotopes (Kastanozem and Chernozem soils) may also act as natural sinks of atmospheric methane. This observation agrees with previous reports regarding methane uptake by unmanaged grasslands [31–33]. However, some unmanaged soils in the steppe region (Solodic Chernozem, Solonetz) contribute to methane emission to Earth's atmosphere.

Among agricultural soils, Chernozem displayed the least methane uptake being −2.2 μg CH_4 m^{-2} h^{-1} only, and Kastanozem showed the highest rate of surface methane consumption reaching −25 μg CH_4 m^{-2} h^{-1}. Overall, the ability of agricultural soils to oxidize atmospheric methane was 3–9 times weaker than in unmanaged soils, and this trend has been demonstrated previously on soils collected in Wales, UK [34]. This is possibly due to the shift from high-affinity methanotrophs to methane oxidizers, known to utilize methane for metabolic purposes in concentrations higher than its atmospheric level. Similar trend has been reported for Brazilian ferralsols by Dorr and colleagues [35]. It has been also demonstrated that extensive NPK and PK fertilizations may result in a two- to three-fold drop in methane oxidizing activity in gray forest soils [36]. Our results support these observations, and we found agricultural soils to be characterized by decreased methane-consuming ability in comparison to unmanaged ones. One of the possible explanations for this observation is that nitrogen fertilizers (in forms of nitrate and ammonium), commonly used in Russia for agricultural purposes, may inhibit the process of methane oxidation due to competitive and noncompetitive inhibition (see [37]).

Maximum potential methane oxidation rates were recorded for unmanaged Podzoluvisol and Luvisol being 1.21 and 1.51 ng CH_4 g^{-1} day^{-1} (Table 2). We found that only a small portion of labeled methane was oxidized during the experiment. More specifically, the ratio did not exceed 21% in unmanaged Luvisol, where the most active methane consumption was detected. Phaeozem and natural solodic Chernozem showed methane oxidation rates of about 0.5 ng CH_4 g^{-1} day^{-1}. In comparison to their unmanaged counterparts, agricultural soils showed lower rates of aerobic methane oxidation ranging from 0.13 to 0.4 ng CH_4 g^{-1} day^{-1}. The only exception was unmanaged Solonetz, which showed extremely low level of potential methane oxidation, 0.04 ng CH_4 g^{-1} day^{-1}, possibly due to unfavorable conditions (both hydrology and salinity) for methanotrophs development. Overall, results of potential methane oxidation rates are consistent with methane uptake values showing decreased ability of agricultural soils to oxidize methane.

Table 2. Potential methane oxidation of the investigated soils.

Soil type	Methane oxidation rate, ng CH_4 g^{-1} day^{-1}		Potential methane oxidation rate, ng CH_4 g^{-1} day^{-1}
	CH_4-CO_2	Biomass incorporation	
Podzoluvisol (natural)	0.70 ± 0.10	0.51 ± 0.06	1.21
Podzoluvisol (agricultural)	0.28 ± 0.10	0.12 ± 0.08	0.40
Luvisol (natural)	0.78 ± 0.17	0.72 ± 0.06	1.51
Luvisol (agricultural)	0.21 ± 0.12	0.13 ± 0.09	0.34
Phaeozem (natural)	0.50 ± 0.08	0.04 ± 0.03	0.54
Solodic Chernozem (natural)	0.38 ± 0.03	0.14 ± 0.07	0.52
Solonetz (natural)	0.04 ± 0.00	0.00	0.04
Chernozem (natural)	0.27 ± 0.04	0.06 ± 0.03	0.33

Table 2. *Cont.*

Soil type	Methane oxidation rate, ng CH_4 g^{-1} day^{-1}		Potential methane oxidation rate, ng CH_4 g^{-1} day^{-1}
	CH_4-CO_2	Biomass incorporation	
Chernozem (agricultural)	0.17 ± 0.02	0.01 ± 0.01	0.18
Gleyic Kastanozem (natural)	0.07 ± 0.01	0.00	0.07
Kastanozem (natural)	0.21 ± 0.02	0.09 ± 0.04	0.30
Kastanozem (agricultural)	0.10 ± 0.03	0.03 ± 0.01	0.13

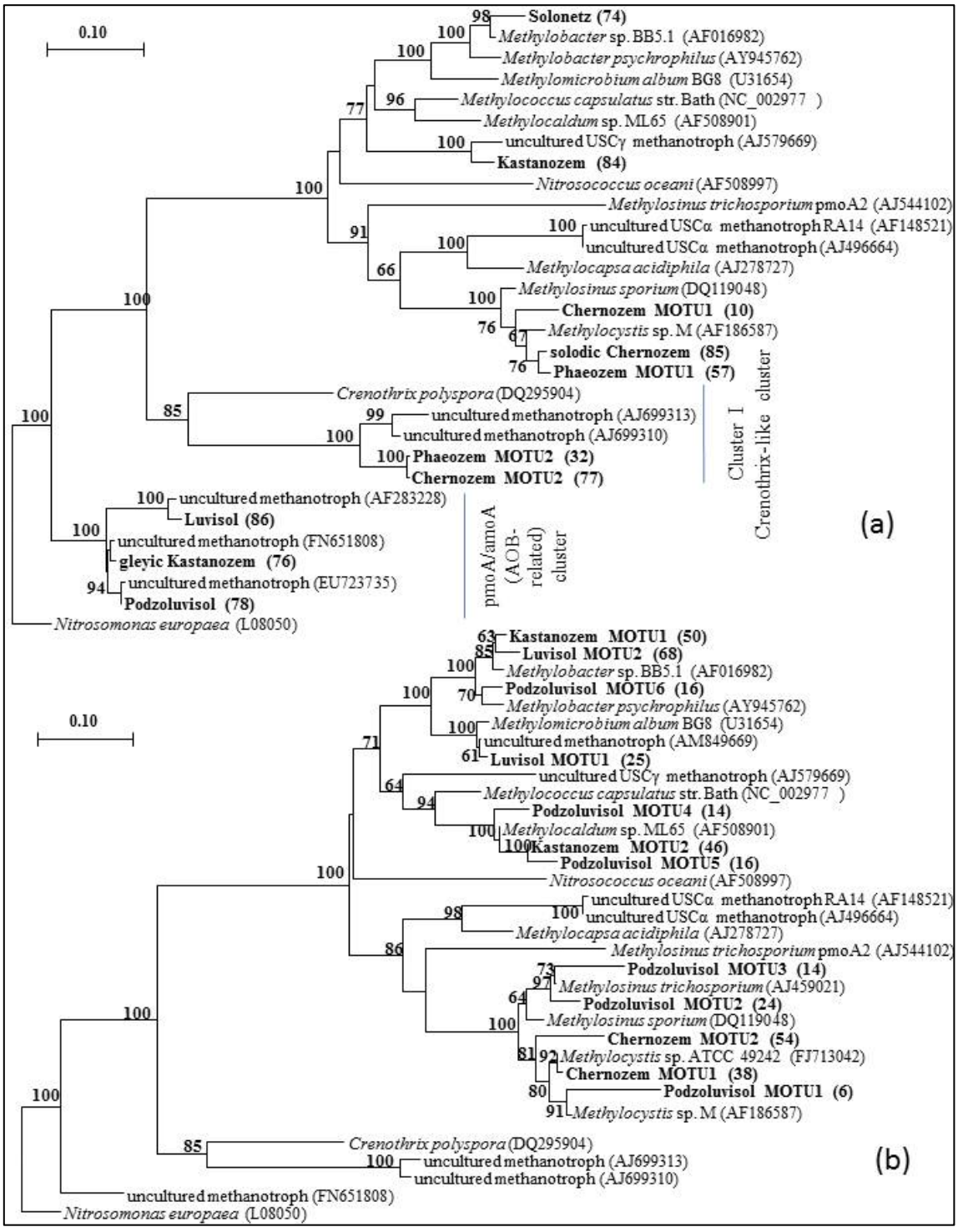
3.2. Diversity of Methanotrophs in Unmanaged and Managed Soils

The primer system A189/A682, used in this study is known to target both methane (*pmoA*) and ammonia monooxygenase (*amoA*) gene fragments due to similarity of hydroxylase components of these two enzymes [38]. Ammonia-oxidizing bacteria bearing *amoA* gene were not in the scope of this study even though the ability of *Nitrosomonas europaea* and *Nitrosococcus oceanus* to oxidize methane in pure culture was reported before [39]. No amplification product for soluble methane monooxygenase (sMMO) was obtained for any of the investigated soils, even though these primers amplify sMMO from *Methylocella*, *Methylomonas*, *Methylosinus*, and *Methylocystis* methanotroph cultures [21]. We assume that sMMO-possessing methanotrophs were either not present, or their abundance was below detection limit in investigated soils. The same tendency was shown for German forest soils by Degelmann and colleagues [40]. Moreover, since pMMO is found in almost all known methanotrophic bacteria [41], and cells expressing pMMO might have a competitive advantage at lower methane concentrations [42], we believe that the analysis, targeting the beta-subunit of pMMO does not limit substantially the validity of our results.

Sequences corresponding to *pmoA2* gene were not found in our clone libraries, possibly due to the fact that this gene is known to be poorly amplified by primers A189/A682 [43]. However, in our previous studies, we have successfully amplified *pmoA2* from methane-oxidizing enrichments [44], where the amount of this gene was high enough. We assume that the contribution of *pmoA2* to methane oxidation during our experiments was rather low.

Methanotrophic communities appeared to be substantially less diverse in unmanaged soils than in soils subjected to agriculture (Figures 2 and 3). Methane oxidizers in natural Podzoluvisol, Luvisol, and gleyic Kastanozem were distantly related to uncultured methanotrophs from Hawaiian forest soil [45], rice field soils [46], and soils in Greenland [47]. The novel *pmoA*/*amoA* cluster detected in the investigated soils was distantly related to known *pmoA* or *amoA* genes (Figure 2). Their phylogenetic position remains debatable and the lack of living cultures belonging to this cluster complicates their phylogenetic placement. Although it might be not completely clear whether they represent *pmoA* of methanotrophs, *amoA* of ammonia-oxidizing bacteria, or genes of enzymes with unique novel functions [48], this group was the only one detected in investigated soils with pronounced ability to oxidize ambient concentrations of methane. According to current knowledge, these methanotrophs are distantly related to *Crenothrix polyspora* and methanotrophs from pH-neutral arctic tundra soils [49]. These organisms are also referred to as "Cluster I" methanotrophs [50].

Figure 2. Phylogenetic placement of amino acids sequences determined in this study; (**a**) unmanaged soils, (**b**) managed soils. The numbers given on branches are frequencies (>60%) with which a given branch appeared in 100 bootstrap replications. The scale indicates the number of expected substitutions accumulated per site. The tree is rooted with *Nitrosomonas europaea*. GenBank accession numbers and number of sequences corresponding to each MOTU are given in parentheses.



Unmanaged Phaeozem and Chernozem harbored methanotrophs, mostly represented by two groups. The first one clusters within methanotrophic Alphaproteobacteria, such as *Methylocyctis* and

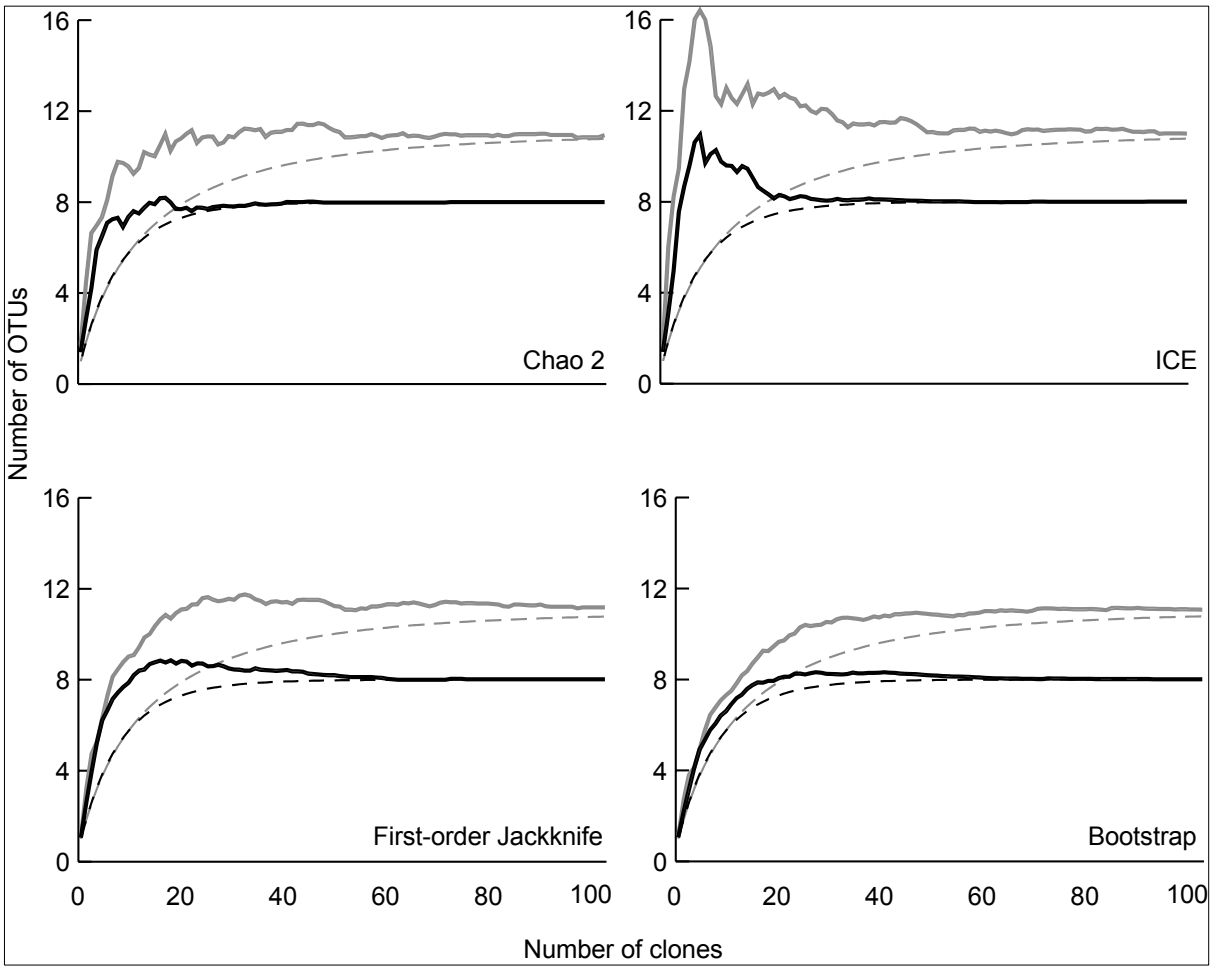
Methylosinus. This group was exemplified in our study by two MOTUs. The first one shows 98% similarity to *Methylocyctis* sp. M, isolated from soil [51], and 95% similarity to *Methylosinus sporium* from Lake Constance [52]. The second MOTU shows 97% similarity to the so-called Cluster I organisms discovered in upland soils [50]. Organisms comprising this group were predominant methane oxidizers in a pH-neutral methane-consuming forest and tundra soils [49], and they are considered to contribute to atmospheric methane oxidation. Although phylogenetic placement of Cluster I remains unclear, some authors report the isolation of methanotrophs (strains K3-16, K3-17) [49] bearing Cluster I *pmoA* genes, suggesting that this type of pmoA (although very divergent) present in some *Methylocystaceae* species [48,49].

The presence of these two groups of methanotrophs, some of which are able to oxidize methane in atmospheric concentrations, explains high methane oxidation rates observed in *in situ* and in laboratory experiments. Analysis of unmanaged Solodic Chernozem and Solonetz yielded one methanotroph in each soil type. The first soil contained the same MOTU as Phaeozem and Chernozem, whereas the second soil harbored methanotroph, showing 97% similarity to estuarine methanotroph *Methylobacter* sp. BB5.1 [53]. Despite substantial differences between the habitats (soil *vs*. estuary), Chernozem, due to its properties, is likely to provide conditions favorable for development of these bacteria, including sufficient moisture, salinity, and neutral pH (Table 1). Unmanaged Kastanozem appeared to be inhabited by methanotrophic bacteria of the USCγ cluster, which were previously found in German Leptosols [54]. We have shown that natural Kastanozem and Gleyic Kastanozem, which are widely spread in southern regions of European Russia, can be a significant sink for atmospheric methane due to being inhabited with putative high-affinity methane oxidizers.

In order to estimate the reliability of biodiversity assessments made within the current study, and to estimate to which extent clone libraries reflect diversity of methanotrophs, we performed clone-based rarefaction analysis. Rarefaction curves reached saturation after approximately 100 clones sampled, and we applied species richness estimators to predict the number of OTUs to be expected from the soils (Figure 3).

All of the applied richness estimators predicted 8 to 11 MOTUs for unmanaged, and 11 to 16 MOTUs for managed biotopes, respectively (data not shown). Remarkably, estimator curves reached saturation starting from 10% to 25% of the analyzed clone library depending on the estimator applied. In other words, analysis of approximately 100 clones is sufficient to assess genetic diversity of methanotrophs detected in the present study. Our results are consistent with earlier studies, which also utilized similar techniques to study methanotrophic communities from deep-sea hydrothermal environments [55], landfill cover soil [56], and glacier forelands [43]. Specifically, it has been shown that hydrothermal deep-sea communities could be sufficiently sampled after the analyzing of only 41 clones [55]. Although this result is far below the sampling effort estimated in our study, it implies that genetic diversity of methanotrophs might be low in some cases, possibly due to the fact that the community structure is very uneven and is characterized by a few dominating species and a large number of minor or rare species. Because approaches based on clone libraries might have certain limitations compared with ones utilizing pyrosequencing, we believe that our experiment has some limitations, including possible undersampling of minor groups. Nevertheless, our results of rarefaction analysis suggest that most of expected methanotrophs have been recovered from analyzed soils.

Figure 3. Estimator-based (solid line) and randomized (dashed line) species accumulation curves for near-natural (black) and managed (grey) beech forests obtained with incidence-based coverage (ICE), Chao 2, first-order Jackknife (Jack 1), and bootstrap richness estimators.



Unlike unmanaged soils, methanotrophic populations in soils collected from agricultural sites appeared to be more species-rich (Figure 3). Podzoluvisol, which has been used to produce barley for about 90 years, yielded six MOTUs, assigned to both to *Alpha*- and *Gammaproteobacteria*. Specifically, we have retrieved *pmoA* sequences related to *Methylosinus* and *Methylocystis* strains, as well as *Methylocaldum* and *Methylobacter* strains (all with 98%–99% similarity). Luvisol under wheat harbored diverse gammaproteobacterial methanotrophs closely related (98% similarity) to *Methylomicrobium album*, which was also detected in agricultural soils [57], uncultured methanotrophs from rice field soils [58], and *Methylobacter* sp. BB5.1 [53]. Managed Chernozem was inhabited by *Methylocystis*-related methanotrophs (99% similarity). For Kastanozem, which was under agricultural use, we detected two methanotrophs assigned to *Methylobacter* sp. BB5.1 (99% similarity) and *Methylocaldum* sp. ML65 (98% similarity), respectively. Overall, our study yielded substantial number of Gammaproteobacteria (Figure 2). This is possibly due to the fact that increased nitrogen availability in agricultural soils (e.g., fertilization) may activate ribosome synthesis in a subset of the overall diversity of methane-oxidizing bacteria, Gammaproteobacteria (Type I methanotrophs) in particular [59,60]. In contrast to unmanaged soils, agricultural sites yielded no *pmoA* sequences,

related to clusters of methanotrophs, which are thought to be responsible for the utilization of atmospheric methane [61]. Our finding that higher methanotrophic diversity is not always accompanied with higher methane oxidation is possibly explained by a difference in methane-oxidizing potential of natural and managed soils.

4. Conclusions

Our study demonstrated that uncultured methanotrophs with pmoA/amoA monooxygenase and Cluster I methanotrophs dominated in methane-oxidizing bacterial communities in unmanaged soils. Furthermore, we showed the contribution of these methanotrophs to the atmospheric methane uptake in both field and laboratory experiments. Thereby, our results highlight the necessity for further studies to be addressed to the studying of these groups.

Methanotrophic population in soils collected from agricultural sites appeared to be more diverse of methane-oxidizing bacteria than in unmanaged soils. However, despite higher numbers of detected MOTUs, managed soils showed decreased methane oxidation rates as observed in both *in situ* and laboratory experiments. In other words, unlike unmanaged soils, soils converted into the agriculture shows substantially reduced capability to convert methane, which is an important greenhouse gas. Our results suggest that natural Kastanozem and meadow Kastanozem, which are widely spread in southern regions of European Russia, can be a significant sink for atmospheric methane. We also found that low-managed abandoned soils display methane oxidation rates comparable with ones recorded for unmanaged areas. Thereby, our results suggest that soil restoration, even if performed within a rather limited time, may have positive effect on methane consumption by terrestrial ecosystems.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Wardle, D.A.; Bardgett, R.D.; Klironomos, J.N.; Setälä, H.; van der Putten, W.H.; Wall, D.H. Ecological linkages between aboveground biota. *Science* **2004**, *304*, 1629–1633.
2. Dick, R.P. A review: Long-term effects of agricultural systems on soil biochemical and microbial parameters. *Agric. Ecosyst. Environ.* **1992**, *40*, 25–36.

3. Birkhofer, K.; Schöning, I.; Alt, F.; Herold, N.; Klarner, B.; Maraun, M.; Marhan, S.; Oelmann, Y.; Wubet, T.; Yurkov, A.; *et al*. General relationships between abiotic soil properties and soil biota across spatial scales and different land-use types. *PLoS One* **2012**, *7*, e43292.
4. Yurkov, A.M.; Kemler, M.; Begerow, D. Assessment of yeast diversity in soils under different management regimes. *Fungal Ecol*. **2012**, *5*, 24–35.
5. IPCC. *Climate Change 2007: Synthesis Report*; Cambridge University Press: Cambridge, UK, 2007.
6. Mancinelli, R.L. The regulation of methane oxidation in soil. *Annu. Rev. Microbiol.* **1995**, *49*, 581–605.
7. Hanson, R.S.; Hanson, T.E. Methanotrophic bacteria. *Microbiol. Rev.* **1996**, *60*, 439–471.
8. Stoecker, K.; Bendinger, B.; Schoning, B.; Nielsen, P.H.; Nielsen, J.L.; Baranyi, C.; Toenshoff, E.R.; Daims, H.; Wagner, M. Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2363–2367.
9. Vigliotta, G.; Nutricati, E.; Carata, E.; Tredici, S.M.; De Stefano, M.; Pontieri, P.; Massardo, D.R.; Prati, M.V.; de Bellis, L.; Alifano, P. *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic γ-Proteobacterium. *Appl. Environ. Microbiol.* **2007**, *73*, 3556–3565.
10. Pol, A.; Heijmans, K.; Harhangi, H.R.; Tedesco, D.; Jetten, M.S.; Op den Camp, H.J. Methanotrophy below pH 1 by a new Verrucomicrobia species. *Nature* **2007**, *450*, 874–878.
11. Op den Camp, H.J.; Islam, M.T.; Stott, M.B.; Harhangi, H.R.; Hynes, A.; Schouten, S.; Jetten, M.S.M.; Birkeland, N.K.; Pol, A.; Dunfield, P.F. Environmental, genomic and taxonomic perspectives on methanotrophic Verrucomicrobia. *Environ. Microbiol. Rep.* **2009**, *1*, 293–306.
12. Dedysh, S.N.; Knief, C.; Dunfield, P.F. *Methylocella* species are facultatively methanotrophic. *J. Bacteriol.* **2005**, *187*, 4665–4670.
13. Vorobev, A.V.; Baani, M.; Doronina, N.V.; Brady, A.L.; Liesack, W.; Dunfield, P.F.; Dedysh, S.N. *Methyloferula stellata* gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase. *Int. J. Syst. Evol. Microbiol.* **2011**, *61*, 2456–2463.
14. McDonald, I.R.; Murrel, C.J. The particulate methane monooxygenase gene *pmoA* and its use as a functional gene probe for methanotrophs. *FEMS Microbiol. Lett.* **1997**, *156*, 205–210.
15. Dumont, M.G.; Murrell, J.C. Community-level analysis: Key genes of aerobic methane oxidation. *Methods Enzymol.* **2005**, *397*, 413–427.
16. Heyer, J.; Galchenko, V.F.; Dunfield, P.F. Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiology* **2002**, *148*, 2831–2846.
17. Schnell, S.; King, G.M. Stability of methane oxidation capacity to variations in methane and nutrient concentrations. *FEMS Microbiol. Ecol*. **1995**, *17*, 285–294.
18. Baani, M.; Liesack, W. Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SC2. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10203–10208.
19. Semenov, V.M.; Kravchenko, I.K.; Kuznetsova, T.V.; Semenova, N.A.; Bykova, S.A.; Dulov, L.E.; Gal'chenko, V.F.; Pardini, G.; Gispert, M.; Boeckx, P.; *et al*. Seasonal dynamics of atmospheric methane oxidation in gray forest soils. *Microbiology* **2004**, *73*, 356–362.
20. Rusanov, I.; Savvichev, A.; Yusupov, S.; Pimenov, N.; Ivanov, M. Exometabolite formation during microbial oxidation of methane in marine ecosystems. *Microbiology* **1998**, *5*, 710–717.

21. Hutchens, E.; Radajewski, S.; Dumont, M.G.; McDonald, I.R.; Murrell, J.C. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. *Environ. Microbiol*. **2004**, *6*, 111–120.
22. Holmes, A.J.; Roslev, P.; McDonald, I.R.; Iversen, N.; Henriksen, K.; Murrell, J.C. Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake. *Appl. Environ. Microbiol.* **1999**, *65*, 3312–3318.
23. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410.
24. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp.* **1999**, *41*, 95–98.
25. Silvestro, D.; Michalak, I. raxmlGUI: A graphical front-end for RAxML. *Org. Divers. Evol.* **2012**, *12*, 335–337.
26. Stamatakis, A. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **2006**, *22*, 2688–2690.
27. EstimateS: Biodiversity Estimations. Available online: http://purl.oclc.org/estimates (accessed on 6 June 2013).
28. Yurkov, A.M.; Kemler, M.; Begerow, D. Species accumulation curves and incidence-based species richness estimators to appraise the diversity of cultivable yeasts from beech forest soils. *PLoS One* **2011**, *6*, e23671.
29. Whalen, S.C.; Reeburgh, W.S. Consumption of atmospheric methane by tundra soils. *Nature* **1990**, *346*, 160–162.
30. Whalen, S.C.; Reeburgh, W.S.; Barber, V.A. Oxidation of methane in boreal forest soils: A comparison of seven measures. *Biogeochemistry* **1992**, *16*, 181–211.
31. Zhou, X.Q.; Wang, Y.F.; Huang, X.Z.; Hao, Y.B.; Tian, J.Q.; Wang, J.Z. Effects of grazing by sheep on the structure of methane-oxidizing bacterial community of steppe soil. *Soil Biol. Biochem.* **2008**, *40*, 258–261.
32. Luo, G.J.; Kiese, R.; Wolf, B.; Butterbach-Bahl, K. Effects of soil temperature and moisture on methane uptake and nitrous oxide emissions across three different ecosystem types. *Biogeosciences* **2013**, *10*, 3205–3219.
33. Mosier, A.; Schimel, D.; Valentine, D.; Bronson, K.; Parton, W. Methane and nitrous oxide fluxes in native, fertilized and cultivated grasslands. *Nature* **1991**, *350*, 330–332.
34. Maxfield, P.J.; Hornibrook, E.R.C.; Evershed, R.P. Acute impact of agriculture on high-affinity methanotrophic bacterial populations. *Environ. Microbiol*. **2008**, *10*, 1917–1924.
35. Dorr, N.; Glaser, B.; Kolb, S. Methanotrophic communities in Brazilian ferralsols from naturally forested, afforested, and agricultural sites. *Appl. Environ. Microbiol*. **2010**, *76*, 1307–1310.
36. Kravchenko, I.K.; Semenov, V.M.; Kuznetsova, T.V.; Bykova, S.A.; Dulov, L.E.; Pardini, G.; Gispert, M.; Boeckx, P.; Van Cleemput, O.; Gal'chenko, V.F. Physicochemical and biological factors affecting atmospheric methane oxidation in gray forest soils. *Microbiology* **2004**, *74*, 255–260.
37. Dunfield, P.F.; Knowles, R. Kinetics of inhibition of methane oxidation by nitrate, nitrite, and ammonium in a humisol. *Appl. Environ. Microbiol.* **1995**, *61*, 3129–3135.

38. Holmes, A.J.; Costello, A.; Lidstrom, M.E.; Murrell, J.C. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiol. Lett.* **1995**, *132*, 203–208.
39. Bedard, C.; Knowles, R. Physiology, biochemistry, and specific inhibitors of CH4, NH4+, and CO oxidation by methanotrophs and nitrifiers. *Microbiol. Mol. Biol. Rev*. **1989**, *53*, 68–84.
40. Degelmann, D.M.; Borken, W.; Drake, H.L.; Kolb, S. Different atmospheric methane-oxidizing communities in European beech and Norway spruce soils. *Appl. Environ. Microbiol*. **2010**, *76*, 3228–3235.
41. McDonald, I.R.; Bodrossy, L.; Chen, Y.; Murrell, J.C. Molecular ecology techniques for the study of aerobic methanotrophs. *Appl. Environ. Microbiol*. **2008**, *74*, 1305–1315.
42. Lontoh, S.; Semrau, J.D. Methane and trichloroethylene degradation by Methylosinus trichosporium OB3b expressing particulate methane monooxygenase. *Appl. Environ. Microbiol*. **1998**, *64*, 1106–1114.
43. Nauer, P.A.; Dam, B.; Liesack, W.; Zeyer, J.; Schroth, M.H. Activity and diversity of methane-oxidizing bacteria in glacier forefields on siliceous and calcareous bedrock. *Biogeosciences* **2012**, *9*, 2259–2274.
44. Kravchenko, I.K.; Kizilova, A.K.; Bykova, S.A.; Menko, E.V.; Galchenko, V.F. Molecular analysis of high-affinity methane-oxidizing enrichment cultures isolated from a forest biocenosis and agrocenoses. *Microbiology* **2010**, *79*, 106–114.
45. King, G.M.; Nanba, K. Distribution of atmospheric methane oxidation and methanotrophic communities on Hawaiian volcanic deposits and soils. *Microbes Environ.* **2008**, *23*, 326–330.
46. Henckel, T.; Roslev, P.; Conrad, R. Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil. *Environ. Microbiol.* **2000**, *2*, 666–679.
47. Barcena, T.G.; Finster, K.W.; Yde, J.C. Spatial patterns of soil development, methane oxidation, and methanotrophic diversity along a receding glacier forefield, Southeast Greenland. *Arct. Antarct. Alp. Res.* **2011**, *43*, 178–188.
48. Kolb, S. The quest for atmospheric methane oxidizers in forest soils. *Environ. Microbiol. Rep*. **2009**, *1*, 336–346.
49. Pacheco-Oliver, M.; McDonald, I.R.; Groleau, D.; Murrell, J.C.; Miguez, C.B. Detection of methanotrophs with highly divergent *pmoA* genes from Arctic soils. *FEMS Microbiol. Lett.* **2002**, *209*, 313–319.
50. Ricke, P.; Kolb, S.; Braker, G. Application of a newly developed ARB-integrated in silico T-RFLP tool reveals the dominance of a novel *pmoA* cluster in a forest soil. *Appl. Environ. Microbiol.* **2005**, *71*, 1671–1673.
51. Uchiyama, H.; Nakajima, T.; Yagi, O.; Tabuchi, T. Aerobic degradation of trichloroethylene at high concentration by a methane-utilizing mixed culture. *Agric. Biol. Chem*. **1989**, *53*, 1019–1024.
52. Bussmann, I.; Rahalkar, M.; Schink, B. Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen. *FEMS Microbiol. Ecol*. **2006**, *56*, 331–344.
53. Smith, K.S.; Costello, A.M.; Lidstrom, M.E. Methane and trichloroethylene oxidation by an estuarine methanotroph, *Methylobacter* sp. strain BB5.1. *Appl. Environ. Microbiol.* **1997**, *63*, 4617–4620.

54. Knief, C.; Lipski, A.; Dunfield, P.F. Diversity and activity of methanotrophic bacteria in different upland soils. *Appl. Environ. Microbiol.* **2003**, *69*, 6703–6714.
55. Nercessian, O.; Bienvenu, N.; Moreira, D.; Prieur, D.; Jeanthon, C. Diversity of functional genes of methanogens, methanotrophs and sulfate reducers in deep-sea hydrothermal environments. *Environ. Microbiol.* **2005**, *7*, 118–132.
56. Cebron, A.; Bodrossy, L.; Chen, Y.; Singer, A.C.; Thompson, I.P.; Prosser, J.I.; Murrell, J.C. Identity of active methanotrophs in landfill cover soil as revealed by DNA-stable isotope probing. *FEMS Microbiol. Ecol.* **2007**, *62*, 12–23.
57. Fjellbirkeland, A.; Torsvik, V.; Øvreås, L. Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmoA*, *mxaF* and 16S rDNA sequences. *Antonie Van Leeuwenhoek* **2001**, *79*, 209–217.
58. Shrestha, M.; Abraham, W.R.; Shrestha, P.M.; Noll, M.; Conrad, R. Activity and composition of methanotrophic bacterial communities in planted rice soil studied by flux measurements, analyses of *pmoA* gene and stable isotope probing of phospholipid fatty acids. *Environ. Microbiol*. **2008**, *10*, 400–412.
59. Bodelier, P.L.E.; Roslev, P.; Henckel, T.; Frenzel, P. Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. *Nature* **2000**, *403*, 421–424.
60. Noll, M.; Frenzel, P.; Conrad, R. Selective stimulation of type I methanotrophs in a rice paddy soil by urea fertilization revealed by RNA-based stable isotope probing. *FEMS Microbiol. Ecol*. **2008**, *65*, 125–132.
61. Dunfield, P.F. The soil methane sink. In *Greenhouse Gas Sinks*; Reay, D., Hewitt, C.N., Smith, K., Grace, J., Eds.; CAB International: Wallingford, UK, 2007; pp. 152–170.