Wetlands (2018) 38:411–422
https://doi.org/10.1007/s13157-017-0984-3

ORIGINAL RESEARCH

Methane Oxidation by Endophytic Bacteria Inhabiting *Sphagnum* sp. and Some Vascular Plants

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Received: 24 November 2015 / Accepted: 28 November 2017 / Published online: 7 February 2018

Abstract
Methane emission from wetlands is responsible for about 24% of the total CH_4 emissions. The value of emission is a result of the balance between the processes of methane formation (methanogenesis) and sinks (methanotrophy). The methanotrophic activity from well-aerated soil surface layers has been relatively well recognized. On the contrary, the active role of plants in reduction of methane emission is rather not fully known. The association of methanotrophic bacteria with plants of *Sphagnum* spp., has already been recognized. In our investigations, particular attention was paid to vascular plants from a peatland overgrown by *Sphagnum* spp. but also *Eriophorum vaginatum*, *Carex nigra*, and *Vaccinium oxycoccos*. The gases emitted from the surface of Moszne peatland were collected using the chamber method from selected sites during growing seasons (spring, summer, autumn). To estimate the contribution of plants in methane emissions from the peatland, in each investigated site gas was sampled from the surface with the native flora cover and after removal thereof. Our results show that the reduction in the CH_4 emission was related to the plant composition, vegetation period, and conditions of the plants. It was confirmed that the endophytes under investigation belonged to type I methanotrophs.

Keywords Methane · Peat soil · Endophytic methanotrophs

Introduction

Methane and carbon dioxide are the main greenhouse gases (IPCC 2013). At the time of the global warming effect, reduction of the methane concentration in the atmosphere, both from natural and anthropogenic sources, is very important. Wetlands, including peatlands, are considered the largest natural source of methane emissions; they emit 100–231 Tg CH_4 into the atmosphere annually, which accounts for 10%–45% of the total emissions of this gas (IPCC 2007). The emission of methane from peatlands is a result of the balance between the processes of formation thereof (methanogenesis) and its sinks (methanotrophy) (Le Mer and Roger 2001) with the latter process being long considered to take place only in the uppermost, well-aerated soil horizons.

The role of plants in wetland methane cycling has been a subject of a two decade-long scientific query (Thomas et al. 1996; Laanbroek 2010; Kip et al. 2012). It was stated that the occurrence of plants such as e.g. *Carex* sp. enhances methane emission by up to 90% (Whiting and Chanton 1992). It was suggested that the increased methane emission is a side effect of plant adaptation to soil anoxia, which includes formation of an internal gas-space ventilation system in stems, roots, and rhizomes aiming to allow oxygen transport to the submerged organs. However, aerenchyma acts as gas conduits not only for O_2. It also creates a shortcut for CH_4 by which it can bypass the aerated soil horizons without being oxidized by methanotrophic bacteria.

Recent discoveries have shown that the carbon cycling in wetlands is far more complicated due to the presence of viable methanotrophic bacteria in the endosphere of *Sphagnum* mosses (Raghoebarsing et al. 2005). It has been demonstrated that methanotrophs inhabiting *Sphagnum* spp., e.g., *Methylocella palustris* and *Methylocapsa acidiphila*, oxidize methane to carbon dioxide, which is later used by *Sphagnum* spp. plants in the process of photosynthesis (Raghoebarsing et al. 2005; Stępniewska et al. 2013). This discovery has substantially

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changed the description of the carbon cycle in peat ecosystems and, at the same time, the global carbon cycle. It has become clear that the methanotrophic endophytes inhabiting plant tissues act as a natural methane filter that can reduce CH_4 and CO_2 emission from peatlands by up to 50% (Kip et al. 2012; Fig. 1). Other field studies have shown the potential ability of the plant–methanotrophic bacteria systems to reduce methane emission up to 77%, depending on the season and the host plant (Goraj et al. 2013).

The role of *Sphagnum* spp. as a host for endophytic methanotrophs has already been a subject of a number of studies (Raghoebarsing et al. 2005; Liebner et al. 2011; Stępniewska et al. 2013; Putkinen et al. 2014). However, mosses are not the only component of the peatland flora. In these ecosystems, a vast array of vascular plants can be found, including numerous species of *Poales* and *Ericales.* Wetland-adapted plants are known to transport soil-produced methane to the atmosphere; these plants also comprise a probable habitat for methanotrophic bacteria. This association however, has not been described so far.

In our investigation, particular attention was paid to *Sphagnum* sp., *Eriophorum* sp., *Carex* sp., and *Vaccinium* sp. - species dominating in the Moszne peatland. The specific research goals were to:

- determine the role that the plant species play in methane emission in situ,
- determine the influence of soil properties on methane emission,
- investigate the presence and activity of endophytic methanotrophic bacteria in plant tissues,
- identify methanotrophic endophytic bacteria

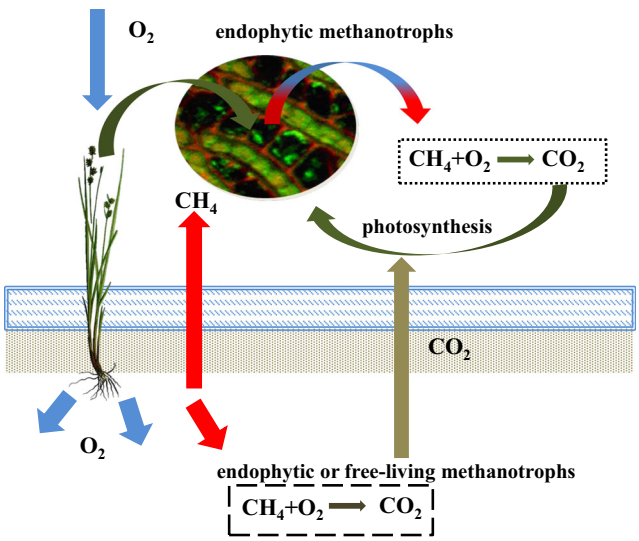

Fig. 1 The role of endophytic methanotrophs in peatlands (modified from Kip et al. 2012)

Material and Methods

Location

The field studies were performed in the area of Moszne Lake, located in the north-western part of the Poleski National Park (PPN) (51° 23′ N, 23° 63′ E) (Fig. 2) in the province of Lublin, in the Polish part of Polesie. The PPN is a part of the West Polesie Biosphere Reserve, protected under the Ramsar convention as an important wetland site with distinguished values of nature. This is a unique territory, being a miniature of tundra at its extremely south-western European location. The climate of the study site is continental with average air temperatures of −4.1 and 17.9 °C in January and July, respectively, and average annual total rainfall of 551 mm (Kaszewski 2002).

The field studies were performed in two locations, characteristic of the Moszne Lake area: a transitional moor (*Sphagno-Caricetum rostratae*) and a continental swamp forest *(Vaccinio uliginosi-Pinetum).* The vegetation of the moor was dominated by mosses *Sphagnum magellanicum, S. fuscum*, *S. fallax, Aulacomium palustre, Polytrichum strictum* and *Drosera rotundifolia* with dense colonies of *Vaccinium* species. The forest undergrowth consisted mostly of graminoids such as *Eriophorum vaginatum*, *E. angustifolium*, *Carex rostrate*, *C. nigra*, and *C. gracilis* with addition of *S. magellanicum.*

Experiment Design

The sampling sites were chosen to represent characteristic elements of the plant cover, i.e. those covered by *S. magellanicum*, *V. oxycoccos*, *E. vaginatum,* and *C. nigra*. Field trips were performed over a period from spring to autumn (one measurement per season in three replicates). In situ analysis involved measurements of the basic soil characteristics such as pH, Eh, EC, and water table (Fig. 3). Subsequent laboratory research included more detailed characteristics of the peat soil (degree of decomposition, TOC) as well as determination of the activity and identity of the methanotrophic endophytic bacteria with the use of molecular biology tools.

Soil Analysis

In Situ Measurements Soil reaction (pH), redox potential (Eh), and electrolytic conductivity (EC) were determined potentiometrically using a multifunctional potential meter pIONneer 65 (Radiometer Analytical S.A., France) equipped with electrodes: a glass electrode (Cartrode pH E16M340) for pH, a combined platinum and Ag/AgCl (reference) electrode (E31M004) for Eh, and a conductivity cell (CDC 30 T-3, Radiometer Analytical S.A., France) for EC

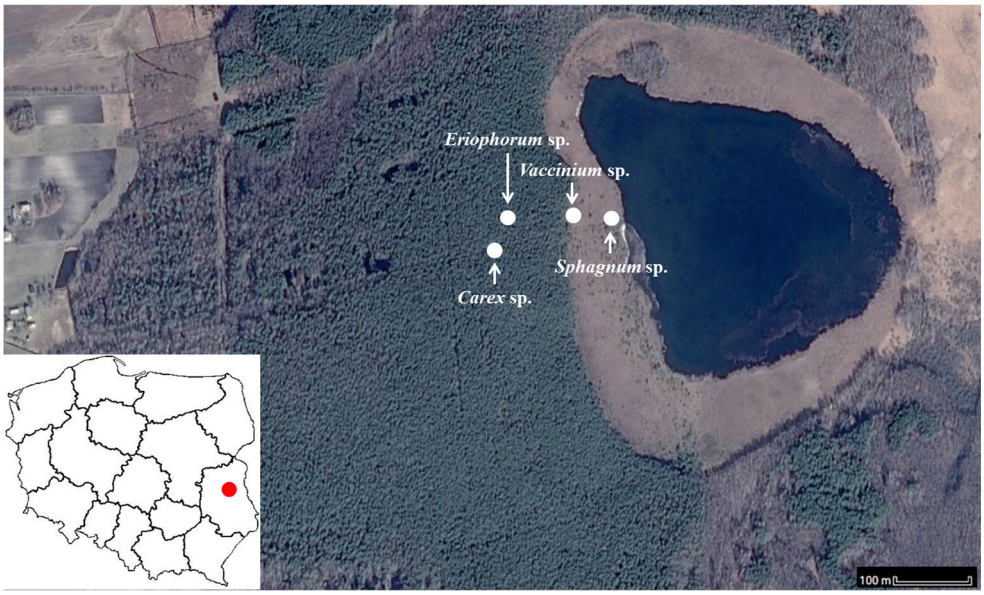

Fig. 2 Localization of the Moszne Lake. Marked places indicate the location of plant and gas sampling

(Bennicelli et al.; 2006; Malawska et al. 2006). The final Eh was corrected for pH values according to a pattern developed by Bennicelli et al. (2006).

Laboratory Determinations of the Peat Soil The degree of peat decomposition was determined using the microscopic method (Fuchsman 1980), while the soil moisture content was estimated by sample weighing after 48-h (105 °C) oven-drying (Gnatowski et al. 2010; Szafranek-Nakonieczna and Bennicelli 2010). The organic matter content (OM) was calculated by loss-on-ignition during combustion of the peat at 550 °C, (Zaccone et al. 2007; Kechavarzi et al. 2010). Organic carbon was determined in water extracts (dissolved organic carbon, DOC) and dry peat samples (total organic carbon, TOC) by combustion and analysis of evolved CO_2 by means of TOC-V_{CSH} with an SSM-5000A module (Shimadzu, Japan).

Methane Emissions

Methane emission was estimated using the static chamber method. Chambers composed of a solid aluminium ring and a PLEXIGLAS-cylinder (0.04516 m^2) (Fig. 4) with a hermetically closed top border were set in locations dominated by the selected plant species. The gas samples were collected after 0 (control) and 60 min through rubber septa placed at the top of the chamber using a gas-tight syringe, transferred to vented vials (20 ml), and analysed by a gas chromatograph (SHIMADZU, GC 2010) equipped with a flame ionization detector (FID), after CH_4 calibration. In each investigated site, gas was sampled from the surface covered by the native flora and after removal thereof. Vascular plants (*Vaccinium* sp., *Carex* sp. and *Eriophorum* sp.) were removed manually, and the opening was thoroughly sealed. In case *Sphagnum* sp.- only the living plant parts were (drawing out, cutting).

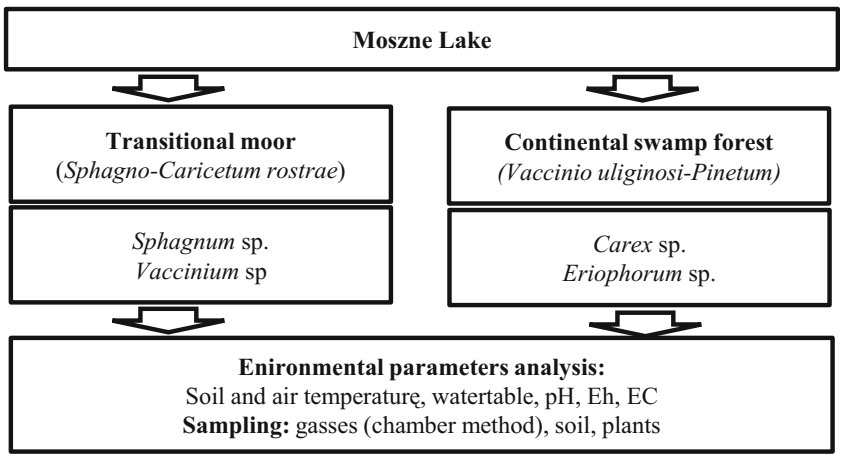

Fig. 3 The scheme of the field research

Fig. 4 Cylinder used for field gas sampling. A - metal ring, B – water-filled thorough, C – plexiglass chamber

Determination of Endophytic Activity

Collection of Plants Plants for the determination of endophytic activity were collected from the area covered with the Plexiglas cylinders after gas sample collection. Complete (as far as possible underground and aboveground parts were collected) live specimens were extracted from soil and immediately transported to the laboratory.

Determination of Endophytic Methanotrophic Activity The methanotrophic activity (MA) of bacteria connected with plants was determined for aboveground and underground plant parts. Whole plants were washed tree times in distilled water, cut into parts: roots and aboveground parts (upper stalk and lower stalk), and immediately placed in glass bottles (120 cm^3) under an atmosphere enriched with 10% *v*/v CH_4 (Praxair, Poland). To each bottle, 5 ml of sterile water to keep plant fragments in good condition. The weight of each sample was about 3 g. Incubations were performed at a temperature of 10 (for spring and autumn specimens) and 20 °C (for plants collected in summer) and normal light conditions (with respect to daily changes) ($n = 3$). The headspace concentrations of gases (CH_4, CO_2, O_2, N_2) were analyzed by a gas chromatograph (SIMADZU, GC 2010) equipped with a flame ionization detector (FID) and a thermal conductivity detector (TCD), after CH_4, CO_2, O_2, and N_2 calibrations. The methanotrophic activity of the plant samples (μM CH_4 g DW^{-1} day^{-1}) was calculated from the slope of the linear regression of CH_4 concentration vs. time ($r^2 \geq 0.95$). Incubations were carried for at least 35 days. Each gas dynamic included 5 or more points.

Identification of the Methanotrophic Endophytic Bacteria

Methanotroph Isolation Methanotrophic bacteria were isolated from the most active part of particular plants by adding surface-sterilized plant fragments to agar mineral medium (NMS) (Whittenbury et al., 1970) and incubated with methane (10% *v*/v CH_4) at 30 °C. Single colonies were first streaked on new NMS agar media. After 14 days, the endophytic methanotrophs were transferred to liquid NMS medium and incubated under the following conditions: a temperature of 30 °C and agitation at 180 rpm, with 10% *v*/v CH_4 in the air.

DNA Isolation Bacterial DNA was isolated from cultures with the method of Sambrook et al. (1989) with own modifications. Cultured cells were harvested by centrifugation and subjected to lysis using a GES solution (5 M *g*uanidine thiocyanate, 100 mM EDTA, 0.5% sarcosyl [pH 8]). DNA was purified using an ice-cold solution of ammonium acetate (7.5 M) and, subsequently, a chloroform:isoamyl (24:1) mixture. Cell debris was removed by centrifugation. DNA was precipitated at -20 °C with isopropanol for two hours, and the pellet was rinsed 5 times with 70% v/v ethanol, dried under vacuum, and resuspended in 20 μl of sterile distilled water.

Polymerase Chain Reaction Amplifications were performed with the use of primers flanking the bacterial 16S rRNA region, namely the 27f/1492R pair (5′-AGAG TTTGATCMTGGCTCAG-3′/5′-TACGGYTACCTTGT TACGACTT-3′), (DeLong 1992). The reaction conditions were as follows: initial denaturation at 96 °C for 4 min 30 cycles of 96 °C for 0.5 min, primer annealing at 55 °C for 1 min, and elongation at 72 °C for 1.3 min. Final elongation was performed at 72 °C for 1 min. The amplification products were analyzed by electrophoresis in 1% agarose gel and stained with ethidium bromide, and visualized in the Red™ Imaging System (Alpha Innotech, San Leandro, USA).

The identity of the methanotrophic bacteria was determined based on sequencing of 16S rRNA gene fragments. The PCR products were sequenced (Genomed S. A., Warsaw, Poland) and compared with sequences stored in NCBI using the BLASTN algorithm. A phylogenetic tree was constructed with the use of the neighbor-joining method with MEGA 6.0. A bootstrap analysis with 1000 trial replications was used to determine the reliability of clustering patterns.

Statistical Analysis

Statistical analyses were performed using Statistica 9 (STATSOFT, USA). The significance of differences between soil characteristics and methane emissions in particular sampling points were tested at the level of $p < 0.05$. Homogeneity of variances and distributions were assessed using Brown-Forsyth and Shapiro-Wilk tests, respectively. Further analysis of the data was performed using parametric (one-way ANOVA) or non-parametric (Kruskal-Wallis or U-Mann Whitney) tests. Pearson or Spearman correlation coefficients were calculated to evaluate the relationship between peat properties and methane emission.

Results and Discussion

Soil Properties

In the investigated sites covered by *C. nigra*, *E. vaginatum*, and *S. magellanicum*, the thickness of peat deposition was found to be 350 cm, while in sites dominated by *V. oxycoccos* it was greater and reached 700 cm. The reaction of peats was usually acidic (2.2 to 5.2). Only in autumn was the soil in the site covered by *S. magellanicum* nearly neutral 6.8. On average, the water table was high, reaching the uppermost soil layer (0–16 cm), and it was lower only in autumn. The Eh potential of the uppermost soil horizon ranged from 451.2 to 644.3 mV (190.1 to 510.0 after pH correction) (Table 1). The oxidation-reduction conditions of the uppermost soil horizon were different depending on the season, as Eh measured in summer was significantly ($p < 0.05$) lower compared to that in the spring and autumn months, measured both directly and after correction to pH 7. Regardless of the seasonal variations, the Eh values suggest that oxygen was freely available in the rhizosphere throughout the vegetation period. The electrical conductivity (EC) in peat was quite low and revealed dependence on both the site and the season. The highest values were found in peat covered by *C. nigra* and *E. vaginatum*, which was especially noticeable in spring and summer (Table 1). This phenomenon probably results from the fact that many *Cyperaceae* species produce dauciform roots. It has been suggested that their function is to acquire P from nutrient-poor, P-fixing soils by means of excretion of compounds such as carboxylates, phenolics, and phosphatases, which facilitate access to the sorbed P (Playsted et al. 2006). The high autumn EC may be explained by the changes in the soil water regimes, as the water table in this part of the year was much lower than in the previous months, thus resulting in a more concentrated soil solution. The organic matter content (OM) was high: above 900 g kg^{-1}, with the highest values in the sites dominated by *S. magellanicum* and *V. oxycoccos*. The sampled sites differed only slightly in the abundance of organic carbon. The measured TOC values were within a narrow range between 53.2 and 56.3% and were slightly higher in peat covered by *C. nigra* and *E. vaginatum*; a similar distribution pattern was found in the case of dissolved organic carbon (DOC, 1.28 to 3.18 g kg^{-1}). However, the degree of peat decomposition was strongly variable; in the site with *C. nigra* and *E. vaginatum*, it was 85% while in the area dominated by *S. magellanicum* and *V. oxycoccos* (moor) it was very low, not exceeding 0.5% (Table 2). Relations between particular soil parameters were summarized in Table 3.

Table 1 Physical and chemical properties of peat soils from the investigated sites - surface layers (0–16 cm)

Season	Plant	pH		Eh (mV)		Eh_7 (mV)		EC (μS)		Watertable (cm)
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Spring	*Carex sp.*	2.9	0.01	497.7	5.3	257.1	5.6	113.8	0.4	0
	Eriophorum sp.	5.2	0.06	444.6	3.5	337.5	2.0	86.0	0.2	−15
	Vacinium sp.	3.2	0.00	602.5	20	375.7	20	57.7	1.5	−15
	Sphagnum sp.	2.7	0.00	644.2	7.6	387.7	7.7	35.5	0.0	−5
Summer	*Carex* sp.	2.6	0.01	451.2	0.7	190.1	0.3	111.0	0.9	0
	Eriophorum sp.	2.9	0.03	651.9	1.2	412.7	1.5	87.1	0.3	−10
	Vacinium sp.	2.6	0.01	489.7	5.7	229.4	5.3	55.8	0.3	−5
	Sphagnum sp.	2.7	0.01	501.2	1.5	246.3	1.0	32.8	0.1	0
Autumn	*Carex* sp.	2.2	0.03	530.5	0.8	244.7	1.6	110.5	0.6	−25
	Eriophorum sp.	5.0	0.00	604.8	1.0	484.6	0.8	30.0	0.0	−40
	Vacinium sp.	2.4	0.03	625.9	3.1	351.9	1.9	193.3	11.5	−60
	Sphagnum sp.	6.8	0.01	519.2	0.7	510.0	0.1	123.5	0.1	−5

Table 2 Characteristics of peat soils from the investigated sites - surface layers (0–16 cm)

Plant	Decomposition degree (%)		OM (g kg $^{-1}$)		TOC (%)		DOC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Carex sp.*	85.5	4.3	906.0	27.8	56.3	0.6	3.18	1.43
Eriophorum sp.*	85.5	4.3	906.0	27.8	56.3	0.6	3.18	1.43
Vacinium sp.**	0.3	0.1	966.3	5.7	53.2	0.5	1.52	0.49
Sphagnum sp.*	0.5	0.1	971.0	1.0	53.2	0.4	1.28	0.28

OM: organic matter; TOC: total organic carbon; DOC: dissolved organic carbon; mineral bedrock depth: * 350 cm, ** 700 cm

Methane Emission

The study was performed in four sites covered by various species of vascular plants and a moss. Methane fluxes from bare soil at the different sites were very variable. Regardless of the season, the highest emissions were detected in the transitional moor, in particular in *S. magellanicum* habitats ($p < 0.05$), where in autumn they reached values as high as 3.052 g CH_4 m^{-2} h^{-2} (Table 3). The lowest flux of the methane was found in locations covered by *E. vaginatum* (0.002 to 0.210 g CH_4 m^{-2} h^{-2}) (Table 4).

The high temporal and spatial variation of CH_4 emission in the air over the investigated sites is a typical feature of the wetland ecosystem (Ding and Cai 2007; Noyce et al. 2014). It has to be noted that methane emission depends upon many physical and chemical factors, e.g. temperature, pH, Eh, etc. (Noyce et al. 2014; Putkinen et al. 2014). Consequently, even in ecologically homogeneous research areas, the coefficient of spatial variation can range from 30% to 100% over short distances (Bartlett and Harris 1993). In the Moszne area, relations between methane emission and soil properties reflected requirenments of the methanogens, e.g. the amount methane emitted grew along with the watertable and OM content. There was also a clear negative relationship with redox potential. Correlation coefficients describing relationships between methane emission as well as between particular properties of the soil were summarized in Table 3.

The parameters described, along with seasonal climatic conditions (e.g. temperature, rainfall), have a considerable influence on the microbial (methanogenic and methanotrophic) activity in the peat (Ding et al. 2004). It has been found that the optimal temperature for both methanogens and methanotrophs is 20–30 °C (Dunfield et al., 2003; Le Mer and Roger 2001). In the current study, the influence of temperature was also visible, especially in the locations covered by *C. nigra* and *V. oxycoccos* where summer emissions were the highest (Fig. 5).

The effect of the plant cover on methane emission was diverse. Among all the sites, only in the location covered by *V. oxycoccos*, the plant cover lead to the decrease in methane emission in each of the investigated seasons, with the highest effectivity in summer (Table 3) (up to 81%). The influence of *C. nigra* and *E. vaginatum* was season-dependent. In spring, both sedges had little influence on methane fluxes from soil to the atmosphere (up to c.a. 9%). In summer, a positive impact of both *C. nigra* and *E. vaginatum* on methane emission was found. This effect, however, did not extend to the autumn months when both sedges were found to reduce methane emission. The influence of *Sphagnum* species was most pronounced in spring, when the plant cover reduced emission significantly, by more than 80%. However, in the subsequent seasons, the effect of moss on methane fluxes was reverse (Table 3). Statistical significance of the described effects was summarized in Tables 5 and 6.

The results obtained in the presented experiment are quite different from those presented by Kölbener et al. (2010), who

Table 3 Coefficients of the correlations between soil parameters and methane emission

Variable	CH_4 emission (gCH4 m^{-2} h^{-1})	pH	Eh (mV)	Eh7 (mV)	EC (μS)	Watertable (cm)	Decomposition degree (%)	OM (g kg $^{-1}$)	TOC (%)
pH	−0.274*	1.000							
Eh (mV)	−0.332*	−0.077	1.000						
Eh7 (mV)	−0.316*	0.696*	0.604*	1.000					
EC (μS)	0.331*	−0.124	−0.124	−0.053	1.000				
Watertable (cm)	0.445*	−0.050	−0.425*	−0.390*	0.069	1.000			
Decomposition degree (%)	−0.687*	−0.215	−0.257	−0.204	−0.815*	0.261	1.000		
OM (g kg $^{-1}$)	0.750*	0.447	−0.123	0.521	0.684*	0.196	−0.617*	1.000	
TOC (%)	−0.721*	−0.316	−0.095	−0.401	−0.801*	0.033	0.644*	−0.927*	1.000
DOC	−0.819*	−0.471	0.165	−0.429	−0.777*	−0.098	0.673*	−0.736*	0.731*

*- significant at $p < 0,05$

Table 4 Influence of vegetation on methane emission in 2011

Samples		Methane emission					
		with vegetation [gCH_4 m^{-2} h^{-1}]		after removal vegetation [gCH_4 m^{-2} h^{-1}]		difference in emissions [gCH_4 m^2 h^{-1}]	contribution of plants to emission [%]*
		Mean	SD	Mean	SD		
Spring	*Carex* sp.	0.252	0.009	0.231	0.013	−0.021	−9.1 ↑
	Eriophorum sp.	0.195	0.038	0.210	0.010	0.015	7.1 ↓
	Vacinium sp.	0.169	0.004	0.225	0.027	0.056	24.9 ↓
	Sphagnum sp.	0.074	0.004	0.528	0.176	0.454	86.1 ↓
Summer	*Carex* sp.	1.084	0.114	0.845	0.050	−0.239	−28.3 ↑
	Eriophorum sp.	0.005	0.002	0.002	0.000	−0.002	−105.2 ↑
	Vacinium sp.	0.097	0.015	0.512	0.111	0.415	81.0 ↓
	Sphagnum sp.	1.469	0.445	0.954	0.282	−0.515	−54.0 ↑
Autumn	*Carex* sp.	0.146	0.009	0.588	0.020	0.442	75.2 ↓
	Eriophorum sp.	0.063	0.004	0.072	0.010	0.010	13.5 ↓
	Vacinium sp.	0.167	0.019	0.735	0.106	0.569	77.3 ↓
	Sphagnum sp.	3.268	0.202	3.052	0.084	−0.215	−7.1 ↑

↑ - vegetation is responsible for the increase in methane emission, ↓ - vegetation is responsible for the decrease in methane emission

*< 0 – enhance emission, > 0 – reduce emission,

investigated peatlands in southern Sweden. Although they also found a link between methane emissions and vegetation covering the study area, the relationship was reverse. It was shown that the total daily CH_4 emission from *E. vaginatum* was 7.42×10^{-3} g m^{-2}, while emission associated with *S. magellanicum* was only 2.204×10^{-3} g m^{-2}. Furthermore, they recorded that the methane emissions from an area covered with *E. latifolium* (broad-leaved cotton grass), *Potentilla palustris* (marsh cinquefoil), *C. rostrata* (beaked sedge), *Anthoxanthum odoratum* (sweet vernal grass), *C. elata* (tufted-sedge), and *C. acutiformis* (lesser pond sedge) were five times higher when compared with the area without plants. These data confirm the active role of the plant cover in CH_4 cycling in the wetland ecosystem. As presented above, depending on the season, the investigated species may both enhance (by providing a transportation route) or diminish methane emission. The latter is possible due to the presence of the active methanotrophic community in the plant endosphere.

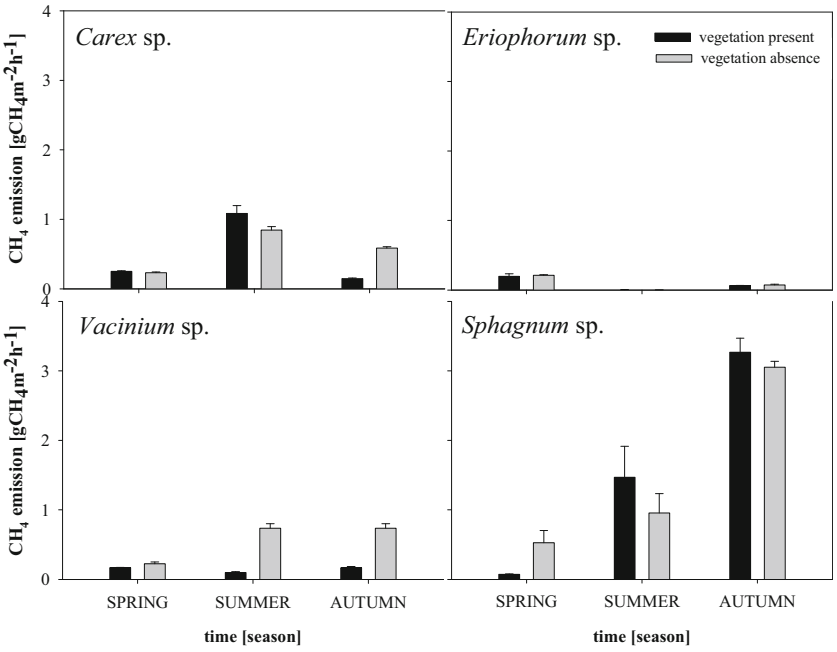

Fig. 5 Averaged methane emission with presence of vegetation and after removal of vegetation in different seasons ($n = 3$)

Table 5 Summary of the results of U Mann-Whitney tests for methane emission with and without plants in particular seasons

Season Plant	Spring	Summer	Autumn
Carex sp.	0.001*	0.001*	0.001*
Eriophorum sp.	0.245	0.006*	0.238*
Vacinium sp.	0.005*	0.005*	0.005*
Sphagnum sp.	0.001*	0.049*	0.006*

In the Moszne peat bog, the highest methane emission from soil was detected in Summer and Autumn (depending on site). It is a well established phenomenon that the density and activity of the endophytic methanotrophic bacteria is positively affected by the intensity of methane flux. In the investigated plots this effect could be seen in sites grown by the *C. nigra* and *V. oxycoccos* (Table 4). Conversely, at the site overgrown by *S. magellanicum*, the plant cover increased methane emission both in the summer and autumn months.

The collected data imply that complex and in-depth investigations are necessary to describe precisely factors that govern methane fate in the plant cover, with particular focus on the endophytic methanotrophic communities. It should be checked e.g. if the composition and activity of the methanotrophic endophytic consortia is related with the condition and/or development stage of a plant (Kim et al., 1999). It has already been reported that the coexistence of bacteria and plants is based on the exchange of metabolic products, which change as the plant grows.

Endophytic Methanotrophic Activity

The methanotrophic activity of the endophytic bacteria measured in laboratory conditions was highly variable. The lowest was observed in *V. oxycoccos* plants, where MA was in the range of 0.199–5.65 μM CH_4 g^{-1} day^{-1}. A slightly higher rate of methane oxidation was found in the two *Cyperaceae* species (0.865 to 22.90 μM CH_4 g^{-1} day^{-1}). The highest MA reaching 67.55 μM CH_4 g^{-1} day^{-1} was determined in incubations containing fragments of the *S. magellanicum* gametophyte (Fig. 6).

Table 6 Statistical significance of the differences between plant impact on methane emission in particular seasons

Plant	Season	Spring	Summer	Autumn
Carex sp.	*Spring*		0.048*	0.048*
	Summer	0.048*		0.001*
	Autumn	0.048*	0.001*	
Eriophorum sp.	*Spring*		0.855	0.544
	Summer	0.855		1.000
	Autumn	0.544	1.000	
Vacinium sp.	*Spring*		0.002*	0.052
	Summer	0.002*		0.838
	Autumn	0.052	0.838	
Sphagnum sp.	*Spring*		0.001*	0.048*
	Summer	0.001*		0.048*
	Autumn	0.048*	0.048*	

The discrepancy between the suggested influence of the plant cover on methane emission and the methanotrophic activity measured in laboratory conditions may be explained by the fact that *V. oxycoccos* specimens were accompanied by a compact layer of *S. magellanicum* mosses, which might have also influenced methane fluxes.

In all the investigated plants, the most active methanotrophic endophytic consortia were found in the specimens collected in summer. Such a tendency may be explained in at least two ways. First, the enhanced methanotrophic activity in summer reflects the shifts in average temperature. The positive impact of the temperature on microbial activity is a well-established phenomenon. Second, the increased summer activity may also result from the fact that, in this part of the year, the tissues of plants are in the optimal stage of development, creating a comfortable niche for bacterial species (Miao et al. 2012).

Analysis of the distribution of MA in particular plant specimens revealed that, in a majority of plants, it was the lowest in the uppermost parts. This may be explained by the fact that in natural conditions the stream of methane from the ground is diluted and a substantial part of the substrate is consumed by the microorganisms located in the roots and lower parts of the stem (in the case of V. oxycoccos and S. magellanicum) or leaves (in the case of Poale C. nigra and E. vaginatum).

Identification of the Endophytic Methanotrophic Bacteria

Analysis of the 16S rRNA sequences revealed that the endospheres of the investigated plants were inhabited by type I γ-Proteobacterial methanotrophs. Isolates obtained from *C. nigra*, *E. vaginatum*, and *S. magellanicum* were similar and closely related to representatives of the genus *Methylomonas* sp. (99% to 96% identity) (Fig. 7). Isolates of *C. nigra* were closely related to the strains found previously for example in the root of *Acorus calamus var. angustatus* (AB683103) (95% identity). Similar bacteria were also found in marine sediments (M95658.1) and wastewater (KJ081955). The endophytes of *E. vaginatum* and *S. magellanicum* were similar to *Methylomonas* sp. isolated from wastewater treatment plant (FR798960, FR798952, FR798959.1) and sediments (AF150806, DQ119049, DQ119049.1, AF150794.1, AM489704.1). *V. oxycoccos* endophytes differed from those described above, as their

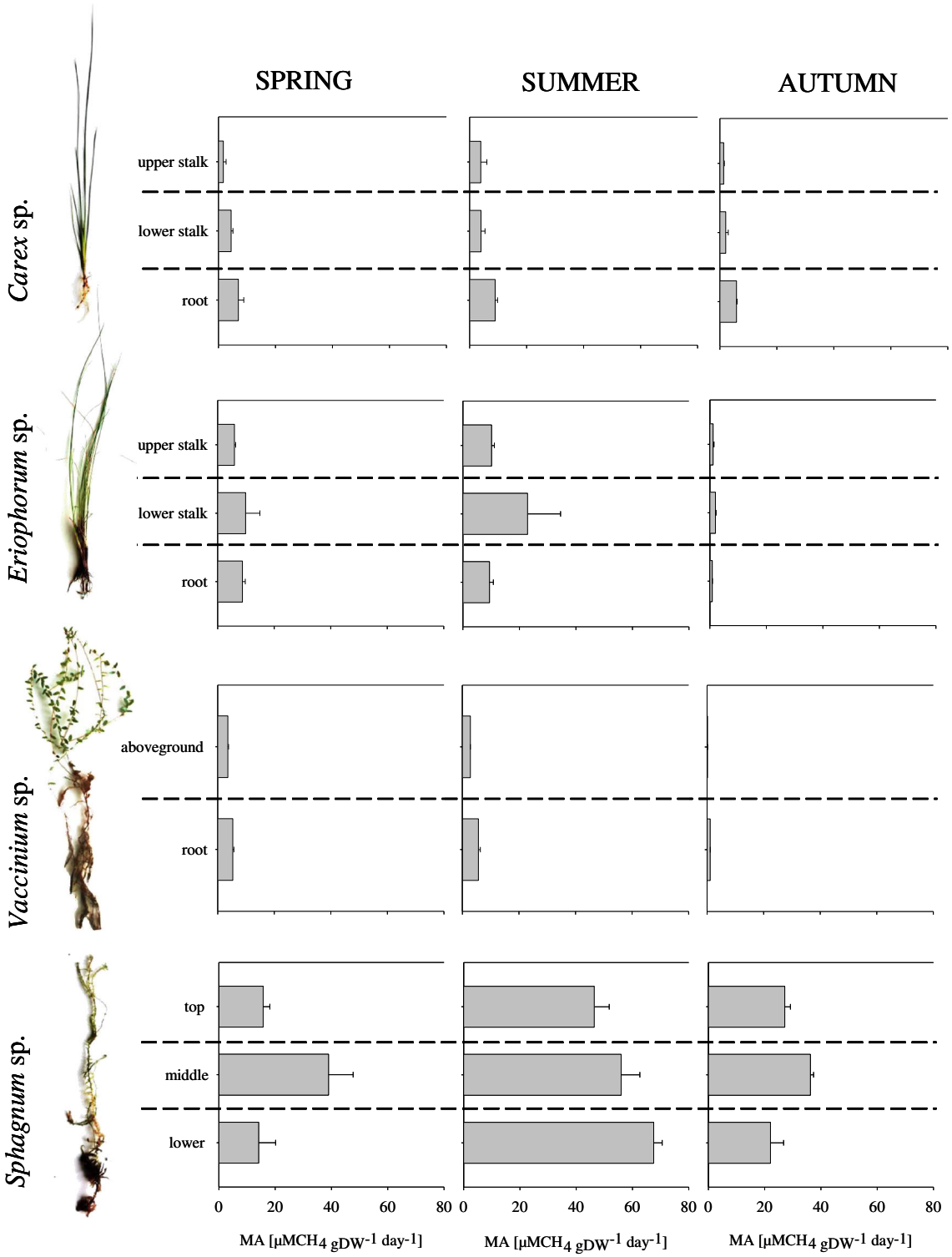

Fig. 6 Methanotrophic activity of the plant species

closest relatives were identified as belonging to the genera *Methylobacter* and *Methylosarcina* (99% identity). These methanotrophs were closely related to the strains isolated from lake sediments (NR_042712, AY007295) (Table 7).

The results obtained are in agreement with reports published previously by Stępniewska and Kuźniar (2014) as well as by Iguchi et al. (2012), who have found type I methanotrophs to be associated with wetland vegetation represented by mosses (*Sphagnum* sp.) and selected vascular

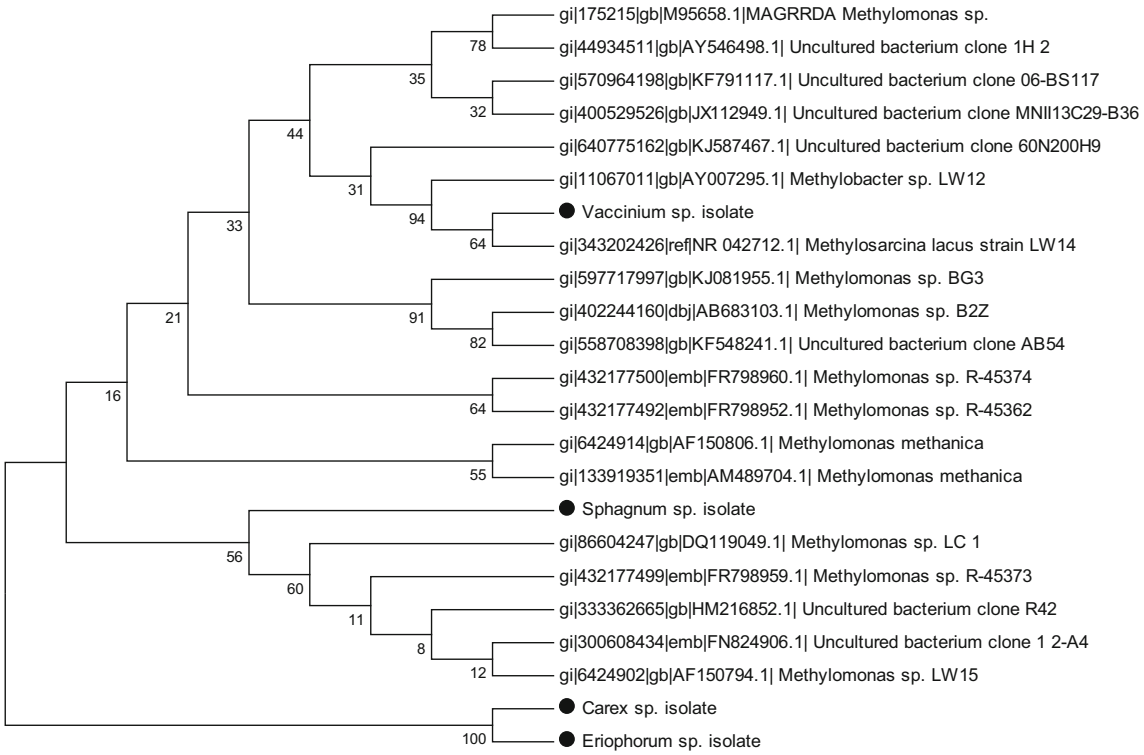

Fig. 7 Phylogenetic tree based on bacterial 16S rDNA sequences. Data with gaps were removed after alignment by CLUSTAL W. The rooted tree was constructed using the neighbor-joining method (Saitou and Nei 1987) contained in the MEGA 5.2 software. Bootstrap values expressed as percentages of 1000 replications are given at branching points (Felsenstein 1985). Bar indicates 10% sequence divergence

Table 7 The closest matches and origin of these matches, from BLASTn queries between 16S rDNA sequences and the GenBank Nucleotide Database for each PCR product

Host	Description	E-value	Identity	Accession	Location
Carex sp.	*Methylomonas* sp. strain A4.	0.0	96%	M95658.1	marine sediment
	Methylomonas sp. BG3	0.0	96%	KJ081955	wastewater
	Methylomonas sp. B2Z	0.0	95%	AB683103	root
	Uncultured bacterium AB54	0.0	94%	KF548241	wastewater treatment plan
	Uncultured bacterium 1H_2	0.0	91%	AY546498.1	lake sediments
Eriophorum sp.	*Methylomonas* sp. R-45374	0.0	97%	FR798960	waste water treatment plant
	Methylomonas sp. R-45362	0.0	97%	FR798952	waste water treatment plant
	Uncultured bacterium, 1_2-A4.	0.0	97%	FN824906.1	biofilm in a treatment system
	Methylomonas methanica	0.0	97%	AF150806	lake sediments
	Methylomonas sp. LC 1	0.0	97%	DQ119049	littoral sediment
Vacinium sp.	*Methylobacter* sp. LW12	0.0	99%	AY007295	freshwater lake sediment
	Uncultured bacterium 06-BS117	4e-58	99%	KF791117	deep-water sponges
	Methylosarcina lacus strain LW14	4e-58	99%	NR_042712	Lake Washington sediments
	Uncultured bacterium MNII13C29-B36	4e-58	99%	JX112949	rice soil
	Uncultured bacterium 60N200H9	4e-58	96%	KJ587467	rice soil
Sphagnum sp.	*Methylomonas* sp. R-45373	0.0	99%	FR798959.1	waste water treatment plant
	Uncultured bacterium R42	0.0	99%	HM216852.1	profundal lake sediment
	Methylomonas sp. LC 1	0.0	99%	DQ119049.1	littoral sediment
	Methylomonas sp. LW15	0.0	98%	AF150794.1	Lake Washington sediment
	Methylomonas methanica VAS23	0.0	98%	AM489704.1	marine sediment

Table 8 Concentrations of bioavailable N and P [mg/kg] in various environmental samples and in Moszne area

Sampling site	$N\text{-}NO_3$	$N\text{-}NH_4$	Reference
Farmland soil	5.3	4.8	Merino et al. 2004
Pasture	6.2	11.6	Merino et al. 2004
Forest	1.9	6.5	Merino et al. 2004
Fertilized pasture	3–22	2–7	Quinn et al. 2007
Natural soil	1–10	0.3–4.6	Shepherd et al. 2001
Oligortophic lake	0.07–0.29	–	Golebiowska et al. 1996
Moszne area	4–7	45–117	unpublished
	$P\text{-}PO_4$		
Natural soil	4.45–8.01		Barajas et al. 2001
Farmland soil	10.51–19.94		Barajas et al. 2001
Peat	73–94		Zhou et al. 2003
Farmland soil	125		Jordan 2003
Oligortophic lake	0.06–0.07		Golebiowska et al. 1996
Moszne area	97–134		unpublished

plants (*Phragmithes australis*, *Acorus calamus var. angustatus*), respectively. Type I methanotrophs are also characteristic representatives of the wetland soil microflora (Wartiainen et al. 2003; Graef et al. 2011; Yun et al. 2012) and are known to be associated with aquatic macrophytes (Yoshida et al. 2014). The prevalence of type I methanotrophs in the wetland ecosystem may be explained by the differentiation of the ecological niche of type I and type II methanotrophs. There is some indication that type I methanotrophs require not only methane and oxygen for growth, but also high levels of other nutrients (Bodelier et al. 2000; Noll et al. 2008), whereas type II methanotrophs are less demanding and therefore have an advantage when nutrients are limited (Graham et al. 1993). This corresponds with the fact that the Moszne peat-bog is a nutrient-rich environment. The concentrations of bioavailable N found in the sampling sites was in the same range as in e.g. agriculturally used soils and several times higher than in the typical oligotrophic environment (Table 8).

Conclusions

Influence of particular plant species on methane emission depends on development stage of a plant, which was the most visible in case the investigated *Cyperaceae* which in the summer enchanced methane emission, most likely via aerenchymal channels.

Endophytic methanotrophs can be found in both underground and emerged plant parts and reveal the highest activity in summer months when both temperature and niche (plant endosphere) development are optimal.

Identity of the endophytic methanotrophs depends on plant species with *Sphagnum* and *Cyperaceae* being inhabited by *Methylomonas*-, while *V. oxycoccos* by *Methylobacter*-like species.

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