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2017-11

Halmeenmäki , E , Heinonsalo , J , Putkinen , A , Santalahti , M , Fritze , H & Pihlatie , M
2017 , ' Above- and belowground fluxes of methane from boreal dwarf shrubs and *Pinus sylvestris* seedlings ' , Plant and Soil , vol. 420 , no. 1-2 , pp. 361-373 . <https://doi.org/10.1007/s11104-017-3406-7>

<http://hdl.handle.net/10138/307478>

<https://doi.org/10.1007/s11104-017-3406-7>

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2 **Above- and belowground fluxes of methane from boreal dwarf shrubs and *Pinus sylvestris* seedlings**

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14 **Keywords**

15 methane flux, methanogens, methanotrophs, boreal forest, plants

16 **Abstract**

17 **Aims**

18 The contribution of boreal forest plants to the methane (CH₄) cycle is still uncertain. We studied the above- and
19 belowground CH₄ fluxes of common boreal plants, and assessed the possible contribution of CH₄ producing and
20 oxidizing microbes (methanogens and methanotrophs, respectively) to the fluxes.

21 **Methods**

22 We measured the CH₄ fluxes and the amounts of methanogens and methanotrophs in the above- and
23 belowground parts of *Vaccinium myrtillus*, *Vaccinium vitis-idaea*, *Calluna vulgaris* and *Pinus sylvestris* seedlings
24 and in non-planted soil in a microcosm experiment.

25 **Results**

26 The shoots of *C. vulgaris* and *P. sylvestris* showed on average emissions of CH₄, while the shoots of the
27 *Vaccinium* species indicated small CH₄ uptake. All the root-soil-compartments consumed CH₄, however, the
28 non-rooted soils showed on average small CH₄ emission. We found methanotrophs from all the rooted and
29 non-rooted soils. Methanogens were not detected in the plant or soil materials.

30 **Conclusions**

31 The presence of plant roots seem to increase the amount of methanotrophs and thus CH₄ uptake in the soil.
32 The CH₄ emissions from the shoots of *C. vulgaris* and *P. sylvestris* demonstrate that the plants have an
33 important contribution to the CH₄ exchange dynamics in the plant-soil systems.

34

35 Introduction

36 Atmospheric methane (CH_4) mixing ratio has increased 1.5-fold since pre-industrial times, and continues to rise
37 with increasing rate (Hartmann et al. 2013). Among land ecosystems the largest known CH_4 sources include
38 anaerobic environments such as natural wetlands and rice paddies, which have an abundance of CH_4 producing
39 archaea (methanogens). Aerated upland soils are the largest sink of CH_4 in the biosphere due to CH_4 oxidizing
40 bacteria (methanotrophs) in the soil (Kirschke et al. 2013).

41 Vegetation is a potentially important factor in the ecosystem-atmosphere CH_4 exchange, and its contribution to
42 the global CH_4 budget is the most uncertain component among all natural CH_4 sources (Carmichael et al. 2014).
43 The most common method to measure soil greenhouse gas fluxes is the chamber method (Livingston and
44 Hutchinson 1995), which does not allow to separate the contributions of the plants or the soil, or the below-
45 and aboveground parts of the system, to the flux. Furthermore, the soil chamber method does not include
46 trees, which are recently discovered to play a significant role in the ecosystem CH_4 exchange (Rusch and
47 Rennenberg 1998; Pangala et al. 2015; Machacova et al. 2016).

48 The net CH_4 flux between ecosystem and the atmosphere depends on the CH_4 production, consumption and
49 exchange rates. In addition to methanotrophs, upland soils may also contain methanogens (Angel et al. 2012),
50 and therefore upland soils have a capacity to shift from a net sink to a net source of CH_4 , when the soil
51 moisture increases enough (Sjögersten and Wookey 2002; Matson et al. 2009; Shoemaker et al. 2014). Active
52 methanogens have also been detected on the surface of roots of Scots pine (*Pinus sylvestris*) and other
53 common boreal forest trees (Bomberg et al. 2011). Furthermore, during wet autumns the CH_4 emissions from
54 upland boreal forest soils can significantly contribute to the ecosystem scale CH_4 balance (Lohila et al. 2016).

55 The transport of methanogen-produced CH_4 from soil to the atmosphere through aerenchyma tissue in plants
56 is a well-documented process among wetland species such as *Carex* spp. (Whiting and Chanton 1992; Joabsson
57 et al. 1999; Ding et al. 2005) and *Menyanthes trifoliata* (Macdonald et al. 1998), as well as in rice (*Oryza sativa*)
58 (Cicerone and Shetter 1981). Riparian deciduous trees (*Fraxinus latifolia*, *Populus trichocarpa*, and *Salix*
59 *fluviatilis*) (Rice et al. 2010) and wetland trees (*Alnus glutinosa* L., *Fraxinus mandshurica* var. *japonica*, and
60 *Betula pubescens*) (Rusch and Rennenberg 1998; Terazawa et al. 2007; Gauci et al. 2010; Pangala et al. 2015)
61 have also been discovered to have a capacity for CH_4 transport. Recently (Maier et al. 2017a) hypothesized that
62 beeches (*Fagus sylvatica*) growing at upland site can also transport CH_4 via roots. In the boreal region, mature
63 *P. sylvestris* trees emitted CH_4 from both stems and shoots, the emissions from the shoots being an order of
64 magnitude larger than those from the stems (Machacova et al. 2016). Thus, not only herbaceous but also
65 woody species are able to transport CH_4 from soil to the atmosphere.

66 Methanogens living in the plant material have also been suggested as the origin of the CH_4 emissions from
67 plants. Emissions of CH_4 have been measured from stem samples of *P. sylvestris* and silver birch (*Betula*
68 *pendula*), in a laboratory experiment (Mukhin and Voronin 2011). Furthermore, high CH_4 concentrations have
69 been detected inside the stems of standing trees of both deciduous (*Quercus rubra*, *Betula alleghaniensis*,
70 *Betula lenta*, and *Acer rubrum*) and conifer species (*Pinus strobus* and *Tsuga canadensis*) (Covey et al. 2012). In
71 addition, during the past decade there have been studies demonstrating that terrestrial plants can also emit
72 CH_4 from living plant material under aerobic conditions (Keppler et al. 2006; Vigano et al. 2008; Bruhn et al.
73 2009; Bloom et al. 2010). Leaf CH_4 emissions from terrestrial plants under aerobic conditions have been
74 demonstrated to be induced by ultraviolet (UV) radiation (Vigano et al. 2008; Bruhn et al. 2009). In contrast,
75 Sundqvist et al. (2012) found CH_4 uptake by the shoots of common boreal trees in the field, including *P.*
76 *sylvestris*, which is contrary not only to the last-mentioned results of the laboratory experiments, but also to
77 the field measurements by Machacova et al. (2016). This underlines the need for further experiments on the
78 plant CH_4 fluxes.

Even though estimates of the contribution of the plants to the ecosystem scale CH₄ flux have been made e.g. for tropical and temperate zones (Pangala et al. 2013; Pangala et al. 2015), the process understanding of CH₄ flux from vegetation is quite limited, and thus vegetation is not yet included in the global CH₄ budget. The production mechanisms behind the CH₄ emissions from plants are not fully understood, and the partitioning of CH₄ fluxes between above and belowground parts of the vegetation is yet unknown. In boreal forest ecosystems the ground vegetation, consisting mainly of dwarf shrubs and mosses, contributes substantially (13%) to the ecosystem gross primary production (GPP) (Kolari et al. 2006; Kulmala et al. 2011). Based on this we hypothesize that ground vegetation may also contribute to the CH₄ exchange in boreal forests.

In this study, we measured CH₄ flux from seedlings of three dwarf shrub species (*Vaccinium myrtillus* L., *Vaccinium vitis-idaea* L., and *Calluna vulgaris* L.) and one tree species (*P. sylvestris* L.) representative to boreal upland forests. The plants were grown in microcosms under controlled laboratory conditions. The aim and uniqueness of this set up was to investigate the roles of the aboveground parts and the belowground root-soil systems of the seedlings on CH₄ fluxes, and to quantify and assess the role of methanogens and methanotrophs in the CH₄ exchange of the studied plants and the soil. Additionally, we compared the flux data to soil chemistry and enzyme activities (Adamczyk et al. 2016), and the number of bacteria in the soil and roots (Timonen et al. 2016), both of which have been analysed within the same microcosm experiment.

Materials and Methods

Experimental design

There were 8 seedlings of each of the four species (*V. myrtillus*, *V. vitis-idaea*, *C. vulgaris*, and *P. sylvestris*) in this experiment, one seedling per microcosm. In addition, there were 11 soil microcosms without a plant, which were treated the same way as the planted ones throughout the experiment. The plants were grown from seeds under laboratory conditions and replanted into the microcosms as small seedlings. The microcosms consist of a thin chamber enclosing the soil and the roots, and of a separate transparent chamber that can be attached to the shoot of a plant (for complete description of the microcosms see Pumpanen et al. (2009)). The total volumes of the soil and the shoot compartments are 348 cm³ and 675 cm³, respectively. The microcosms are not permeable to UV radiation.

The humus layer of the soil and the berries of the *Vaccinium* species were collected in autumn from a Scots pine dominated forest near the SMEAR II station in Hyytiälä, southern Finland (61°51'N, 24°17'E, 181 m a.s.l.), where the soil type is haplic podzol (FAO/UNESCO 1990). The field layer is dominated by *V. myrtillus* and *V. vitis-idaea*, and also *C. vulgaris* is common in the area (Kolari et al. 2006). The soil was homogenized and sieved before using for the construction of germination pots and the microcosms. The seeds of *P. sylvestris* were from a seed lot (M29-92-0059, Natural Resources Institute Finland), and the seeds of the *Vaccinium* species were separated from the berries. All the seeds were surface sterilized before germination (for details see Adamczyk et al. (2016)). *C. vulgaris* seedlings were germinated naturally from the sieved humus. At the time of constructing the microcosms, ca. 178 cm³ of moist homogenized humus soil was inserted into each soil compartment.

Before the CH₄ flux measurements, the seedlings had been in stabile growth conditions for ca. 11 months, and they had grown to a height of ca. 10 cm (for details see Adamczyk et al. (2016)). The growth room was equipped with daylight spectrum lamps, which followed a diurnal rhythm of 18 hours daytime and 6 hours night-time. The daytime photosynthetic photon flux density (PPFD) was 160–220 μmol m⁻² s⁻¹ (LI-190R Quantum Sensor with LI-COR Biosciences LI-250A Light Meter, LI-COR Biosciences, Nebraska, USA) and temperature at 18 °C, and the night-time temperature was at 14 °C. The soils were watered three times a week with reverse osmosis water to 100% water holding capacity. The last irrigation was done approximately 24

hours before the flux measurements. The plants' exposure to daylight lamps was ceased ca. 12 hours before the measurements.

Methane flux measurements

For the flux measurements the soil and the shoot compartments of the microcosms were separately but simultaneously closed airtight, and three manual gas samples of 20 ml were taken from the air space of each compartment at time 1 min, 30 min and 60 min after closure with a plastic syringe (BD Plastipak™, Becton, Dickinson and Company, New Jersey, USA). At the same time with each sampling the same volume (20 ml) of replacement air (compressed air; 2.0 ppm CH₄) was injected into the microcosm in order to maintain the air volume and to avoid pressure changes. Note that the non-planted soil microcosms did not have an aboveground cuvette. The temperature on top of the microcosms was between 16–24.5 °C during the measurements (median 19.5 °C). The sampling was performed in regular indoor light conditions with PPFD ranging between 10–19 μmol m⁻² s⁻¹ and without UV radiation.

Gas samples were injected into glass vials (12 ml, Labco Exetainer®, Labco Limited, Wales, UK) and analysed with a gas chromatograph (7890A, Agilent Technologies, California, USA) equipped with a flame ionization detector (FID) (for details of the instrument see Pihlatie et al. 2013). The method quantification limit (MQL) of the analyser was calculated according to Corley (2003), and it was 0.10 ppm for CH₄.

The CH₄ flux was calculated using the least squares method to calculate the line of best fit for the concentration change in time. The belowground compartment volume used for the flux calculation was quantified as the air space around and within the soil. The porosity was calculated by using the particle density of organic matter of a pine forest (Redding et al. 2005). The soil air within the pores was determined by subtracting the volume of water, which was calculated from the soil fresh weight (FW) and dry weight (DW) and the density of water (1 g cm⁻³). The average air volume of the belowground compartments was 229 cm³. The CH₄ flux was further divided by the total DW of the below- or aboveground material. The flux data was further processed by setting the flux values with NRMSE (Normalized Root Mean Square Error) value larger than 0.35 and R² value smaller than 0.5 to zero. This was based on an assumption that very small fluxes with poor goodness of fit, and measurements with highly fluctuating CH₄ concentrations can be assumed as zero fluxes, which represented 40% of the data. From the fluxes that met the quality criteria, 56% were above the MQL. The MQL is rather conservative, and thus also fluxes smaller than MQL are reported in the results if the data quality passes the NRMSE and R² criteria. The net fluxes of the plant-soil systems were calculated by adding the belowground flux to the aboveground flux (as nmol CH₄ h⁻¹), and dividing this flux by the total DW of soil and plant material.

After the CH₄ flux measurements, the microcosms were dismantled. For the plants, the shoots were cut off, and the roots were carefully separated from the soil. Samples of shoots, roots, and soil were freeze-dried, and thereafter stored at -20 °C. The dried shoots and roots were weighted separately. The FW and DW of the non-rooted soils were determined, and to quantify the total soil weight of the rooted soils, the mean of the non-rooted soil DWs was used.

Microbiological analyses

Total DNA was extracted from the roots, stems and leaves of all the plants, as well as the soil by weighing 0.05 g (FW) of fresh soil, 0.25 g (FW) of fresh roots and 0.025 g (DW) of dry lyophilized and ground shoot material (stem and leaves). Soil samples were extracted with NucleoSpin® Soil genomic DNA extraction kit (Macherey-Nagel, Düren, Germany) and roots were extracted manually from homogenized sample material with hot-CTAB method at +65 °C as in (Timonen et al. 2016). DNA from soil and roots were further purified with PowerClean® DNA Clean-up kit (Mo Bio Laboratories Inc, USA) according to manufacturer's instructions. Shoot samples were extracted using NucleoSpin® Plant genomic DNA extraction kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions without further purification.

To detect and quantify the methanogenic archaea, quantitative PCR (qPCR) with primers mlas/mcrA-rev (Steinberg and Regan 2008) targeting the *mcrA* gene coding for the α -subunit of the methyl-coenzyme M reductase was applied. Analysis was done in 20 μ l reaction volume with 1x SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 500 nm of each primer and 1–10 ng of the template DNA (1 μ l of undiluted or 1:10 diluted DNA extract from root, shoot or soil samples). Amplification was performed with CFX96 Touch™ Real-Time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA) with the following cycling parameters: 98 °C for 3 min, 45 cycles of 98 °C for 15 s and 60 °C for 1 min. To verify the specificity of the products, melt curve analysis was performed by increasing the temperature from 65 °C to 95 °C with 0.5 °C increment per every 5 s. All possibly positive samples were also run in 1.0% agarose gel (Bioline, London, UK), stained with 0.3% (W/w) ethidium bromide (Sigma-Aldrich, Steinheim, Germany) and visualized under UV-light. Negative PCR controls remained free of PCR amplicons. Standard curve was based on a 10-fold dilution series of a target PCR product cloned into a TOPO pCR2.1 plasmid (Thermo Fisher Scientific, Waltham, MA, USA). Standards and samples were run in duplicate in each run. Efficiencies of the qPCR amplifications were between 96.5% and 100.5% in the individual *mcrA* runs. DNA concentrations were measured with Qubit 2.0 Fluorometer with dsDNA HS Assay Kit (Thermo Fisher Scientific).

Methanotrophs were analysed with primers A189f/A650r (Holmes et al. 1995; Bourne et al. 2001) targeting the *pmoA* gene coding for the α -subunit of particulate methane monooxygenase. Reverse primer mb661r (Costello and Lidstrom 1999) was also tested but no proper products were amplified. Protocol for the *pmoA* was analogous to *mcrA* except for the annealing/extension temperature being 59 °C, lower primer amount (300 nM each), addition of 5 μ g of bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA) to the reaction mix and running the standard dilution series in triplicate. Test runs with shoot derived DNA extracts (individual and pooled) did not provide correct *pmoA* PCR product and thus shoots were not thoroughly analysed with *pmoA* qPCR. Efficiencies of the qPCR amplifications were between 88% and 95.3% in the individual *pmoA* runs.

Inhibition of the samples was tested with spiking the standard with template DNA as in Goebel et al. (2010). Inhibition levels were calculated for each plant/sample type and taken into account when calculating the gene copy numbers (on average 5% for soil, 0.7% for roots and 3% for shoots).

Based on all the individual qPCR runs, the smallest reliably quantified standard was 10^1 gene copies reaction⁻¹ (both assays) which was thus considered the limit of quantification. In our samples, this value equals on average 6.7×10^5 gene copies g⁻¹ (DW) for soil, 5.2×10^4 gene copies g⁻¹ (DW) for roots and 4.0×10^4 gene copies g⁻¹ (DW) for shoots, respectively. If the quantification limit was not reached, genes were still considered detected if the PCR product was visible and the size matched the standard product when visualized in 1% agarose gel.

198 **Statistical analyses**

The differences between the mean values of the fluxes from different compartments were tested by analysis of variance (ANOVA), and Tukey-Kramer test was applied as a post hoc test for multiple comparison. The differences between the numbers of methanotrophs was tested with Welch ANOVA due to unequal variances, and Games-Howell post hoc test was used. For both the fluxes and methanotrophs, the differences from zero were tested with one sample t-test. Two-sample t-test was performed to test the differences between two groups, except when the groups had unequal variances, Welch t-test was applied.

The Spearman's correlation was used to study the relationships between the methanotrophs and the CH₄ fluxes. In addition, the CH₄ fluxes of this study were compared to other variables analysed from samples from the same microcosms: soil extracellular enzyme activity, soil organic matter characteristics, and the number of enchytraeid worms in the soil (Adamczyk et al. 2016), and to the number of bacterial 16S rRNA gene copies

(total amount of bacteria) within the soil and the roots (Timonen et al. 2016). The relationships were studied with the Spearman's correlation. To assess the effect of general enzyme activity of the soils, we calculated a sum of all the enzyme activities measured by Adamczyk et al. (2016). The correlation analyses were performed separately for the above- and belowground data, as well as for the different plant species and soil.

All the statistical analyses were assessed at a significance level of $p < 0.05$. The statistical analyses were performed with Matlab (version R2014a, MathWorks, Massachusetts, USA), except for Welch ANOVA and Welch t-test, which were done by using SPSS (version 22, IBM SPSS Statistics, New York, USA).

Results

Methane fluxes

The average CH₄ fluxes from belowground compartments of all the studied plants showed small CH₄ uptake, while the bare soil emitted small amounts of CH₄ (Table 1). The fluxes from the rooted soils were between -2.8 – 1.7 nmol CH₄ h⁻¹ or -0.081 – 0.049 nmol CH₄ h⁻¹ g⁻¹ (DW), and from the non-rooted soils between -1.1 – 3.5 nmol CH₄ h⁻¹ or -0.039 – 0.12 nmol CH₄ h⁻¹ g⁻¹. There was a statistically significant ($p < 0.01$) difference between the mean CH₄ fluxes of the bare soil and the soil with *V. vitis-idaea* roots. Moreover, when all the rooted soils were pooled together and compared to the non-rooted soil, there was a difference between the mean fluxes ($p < 0.01$). Additionally, there was a significant difference between the belowground fluxes of *V. vitis-idaea* and *V. myrtillus*, as well as between *V. vitis-idaea* and *C. vulgaris* ($p < 0.05$). The average aboveground fluxes of *C. vulgaris* and *P. sylvestris* indicated small CH₄ emissions, while the mean flux of the *Vaccinium* species were slightly negative (Table 1). The CH₄ fluxes from the shoot compartments were ranging from -6.6 to 7.3 nmol CH₄ h⁻¹ or -3.2 – 3.3 nmol CH₄ h⁻¹ g⁻¹. The aboveground mean flux was significantly higher than the belowground mean flux when all the species were pooled together ($p < 0.05$).

Table 1. The arithmetic means $\pm$ standard errors of the means, medians, minimum and maximum of the CH₄ fluxes in nmol h⁻¹ and nmol h⁻¹ g⁻¹ (DW of the total plant and/or soil mass), and the number of microcosms in each group. The statistics of the fluxes are given for the whole plant-soil system (net flux), and separately for the belowground (belowg.) and aboveground (aboveg.) compartments.

	CH ₄ Flux (nmol h ⁻¹)				CH ₄ Flux (nmol h ⁻¹ g ⁻¹)				n
	Mean $\pm$ SE	Median	Min	Max	Mean $\pm$ SE	Median	Min	Max	
<i>V. myrtillus</i> (net flux)	-0.14 $\pm$ 1.0	-0.45	-3.9	3.9	-0.0029 $\pm$ 0.028	-0.012	-0.11	0.11	8
Belowg.	-0.025 ^a $\pm$ 0.32	0	-1.2	1.7	-0.0070 ^a $\pm$ 0.0091	0	-0.035	0.049	8
Aboveg.	-0.16 $\pm$ 0.93	0	-3.9	3.9	-0.045 $\pm$ 0.61	0	-3.2	3.0	8
<i>V. vitis-idaea</i> (net flux)	-1.7 $\pm$ 0.46	-2.1	-3.5	0.044	-0.044 $\pm$ 0.012	-0.052	-0.086	0.0012	8
Belowg.	-1.2 ^{b*} $\pm$ 0.41	-1.2	-2.8	0	-0.035 ^{b*} $\pm$ 0.012	-0.036	-0.081	0	8
Aboveg.	-0.46 $\pm$ 0.55	0	-3.5	1.7	-0.028 $\pm$ 0.15	0	-0.71	0.83	8
<i>C. vulgaris</i> (net flux)	3.2 $\pm$ 1.4	3.8	-2.8	7.7	0.084 $\pm$ 0.036	0.10	-0.078	0.20	8
Belowg.	-0.051 ^a $\pm$ 0.24	0	-1.5	0.75	-0.0015 ^a $\pm$ 0.0071	0	-0.044	0.022	8
Aboveg.	3.2 $\pm$ 1.5	4.2	-3.6	7.3	1.1 $\pm$ 0.64	1.2	-2.0	3.2	8
<i>P. sylvestris</i> (net flux)	1.2 $\pm$ 1.5	0.78	-7.2	6.3	0.032 $\pm$ 0.041	0.021	-0.20	0.17	8
Belowg.	-0.35 ^{ab} $\pm$ 0.16	-0.19	-1.2	0	-0.010 ^{ab} $\pm$ 0.0045	-0.0054	-0.035	0	8
Aboveg.	1.6 $\pm$ 1.5	0.97	-6.6	6.7	0.54 $\pm$ 0.61	0.41	-2.7	3.3	8
Non-rooted Soil	0.59 ^a $\pm$ 0.37	0.44	-1.1	3.5	0.019 ^a $\pm$ 0.013	0.011	-0.039	0.12	11

^{ab} Means of the belowground fluxes denoted by a different letter are significantly different ($p < 0.05$)

* Significant difference from zero ($p < 0.05$).

The net fluxes of the seedlings of *C. vulgaris* and *P. sylvestris* indicated on average CH₄ emissions (Table 1.), since the CH₄ uptake of the rooted soils of these two species were relatively small compared to the shoot emissions. The mean net fluxes of the *Vaccinium* species showed CH₄ uptake (Table 1.), while the net uptake of *V. vitis-idaea* was larger than the net uptake of *V. myrtillus* due to significant difference between their average belowground fluxes.

In addition to the fluxes, we examined the CH₄ mixing ratios in the last samples taken after one hour closure. We compared the CH₄ content between the above- and belowground compartments, and between the rooted and non-rooted soils. The measured mixing ratios of CH₄ from the shoot compartments were between 2.0–2.3 ppm, while the root compartment mixing ratios were between 1.6–2.1 ppm. The mixing ratios from the non-rooted soil were at the same level as those from the shoots, ranging between 2.0–2.2 ppm. The mean CH₄ mixing ratios in the shoot compartments were significantly higher than those in the root compartments for all the species ($p < 0.01$) (Fig. 1). Furthermore, the mean CH₄ mixing ratios of the root compartments of all the plant species were significantly lower than the mean of the soil compartments ($p < 0.01$).

Fig. 1 The mean CH₄ mixing ratios (ppm) in the 3rd gas samples after one hour closure from the above- and belowground compartments of different plants and soil. The black circles and grey triangles are the mean values, and error bars are standard error of the mean (SEM). The statistically significant differences ($p < 0.01$) between the compartments are indicated by different letters. The grey line is the mean of the background CH₄ level before the measurements, and the shaded area shows the standard error of the mean (SEM)

Methanotrophs and methanogens

Methanotrophs were detected in quantifiable amounts in the non-rooted soils as well as in the rooted soils of all the studied species (Fig. 2). The rooted soils of all the studied plants contained more methanotrophs than the non-rooted soil, and the difference was statistically significant for *V. myrtillus*, *V. vitis-idaea*, and *C. vulgaris* ($p < 0.05$) (Fig. 2). *V. vitis-idaea* and *C. vulgaris* had the highest amounts of methanotrophs. There was also a difference between the rooted and non-rooted soils when all the species were pooled together ($p < 0.001$). Methanotrophs were also detected in the roots of all plants but their *pmoA* gene copy numbers were below the quantification limit ($< 10^1$ copies reaction⁻¹). No valid *pmoA* products were amplified from any of the shoot samples.

Methanogens were below the detection limit in all of the samples.

Fig. 2 Methanotroph related *pmoA* gene copies in soil (gene copies g⁻¹ of soil DW), based on primer pair A189f/A650r. The black circle is the mean, and the error bars represent the standard error of the mean. The letters indicate statistically significant differences and asterisks show significant differences from zero ($p < 0.05$)

Effects of biotic and abiotic variables on the CH₄ fluxes

All the rooted soils pooled, there were negative correlations between the CH₄ fluxes and the total amount of bacteria in the roots (measured by Timonen et al. (2016)) ($r_s(21) = -0.43$, $p < 0.05$), between the fluxes and the soil recalcitrant nitrogen (N) pool ($r_s(17) = -0.55$, $p < 0.05$), and between the fluxes and the soil NH₄ concentration ($r_s(30) = -0.52$, $p < 0.01$) (all the soil chemical properties were measured by Adamczyk et al. (2016)) (Online Resource 1). A strong negative correlation was found between the *P. sylvestris* belowground CH₄ fluxes and the concentration of total water-soluble phenolic compounds in the soil ($r_s(6) = -0.84$, $p < 0.05$) (Online Resource 1). In addition, there was a strong, although barely non-significant, negative correlation between the *P. sylvestris* belowground flux and the amount of bacteria in the soil ($r_s(4) = -0.88$, $p = 0.050$) (Online Resource 1). A negative correlation denotes that as the explanatory variable increases, the CH₄ flux decreases towards more negative values i.e. the CH₄ uptake increases, and a positive correlation stands for the CH₄ flux increasing towards emission with increasing explanatory variable.

When all the aboveground fluxes were pooled, there were significant positive correlations between the shoot CH₄ fluxes and the total water-soluble phenolic compounds in the soil ($r_s(30) = 0.45$, $p < 0.01$), and between the fluxes and the soil condensed tannins ($r_s(30) = 0.40$, $p < 0.05$) (Online Resource 1). When the shoot CH₄ fluxes were explored one species at a time, there was a strong positive correlation between the shoot fluxes of *V. vitis-idaea* and the total N content in soil ($r_s(6) = 0.85$, $p < 0.05$) (Online Resource 1).

Discussion

The design of this experiment provided a unique opportunity to observe the differences of the CH₄ fluxes between the above- and belowground parts of the plant-soil systems, and study separately plant species that normally grow intermingled in the same habitat. In this study the observed fluxes were also connected with the abundances of the CH₄ consuming and the producing microbes. We discovered that the aboveground parts of *C. vulgaris* and *P. sylvestris* were on average sources of CH₄, while the shoots of the *Vaccinium* species showed on average small uptake. The rooted soils of all the studied species indicated CH₄ uptake, as expected based on

292 general understanding of forest soils (Kirschke et al. 2013). Interestingly, as the soil without a plant showed
293 small CH₄ emission, our data highlights that active plant root systems strongly promote methanotrophy in
294 forest soils. These findings were supported by the differences in the CH₄ mixing ratios between the above- and
295 belowground compartments, and the rooted and non-rooted soils. Based on current understanding the shoot
296 emissions may result from two different mechanisms: 1) transport of soil-derived CH₄ by the plants (Rusch and
297 Rennenberg 1998; Rice et al. 2010; Pangala et al. 2015), or 2) CH₄ production within the plants, which can be
298 either anaerobic and microbial (Mukhin and Voronin 2011), aerobic and microbial (Lenhart et al. 2015) or
299 aerobic and non-microbial (Keppler et al. 2006; Vigano et al. 2008; Bruhn et al. 2009; Bloom et al. 2010).

300 As *mcrA* gene copies, belonging to methanogenic archaea, were below the detection limit in all the plant and
301 soil samples in this study, our results suggest that the CH₄ emitted from the plant shoots was not produced by
302 methanogens. However, the positive CH₄ flux together with the relatively high CH₄ mixing ratio detected in the
303 non-rooted soils suggest that CH₄ production did indeed occur in the soil. In this case, some of the CH₄ may
304 have been transported by the plant from the soil to the aboveground air space, which would also in part
305 explain the difference between the rooted and non-rooted soil CH₄ fluxes. While an aerenchyma formation
306 within ericoid shrubs is yet unknown, some flood-tolerant *Pinus* species have been discovered to form an
307 aerenchyma when grown in hydroponic solution (Topa and McLeod 1986). Although dry forest soils are not in
308 general preferable environments for the methanogens, previous research has observed presumably
309 methanogen-derived CH₄ production in oxic soils (Andersen et al. 1998; von Fischer and Hedin 2002) and
310 revealed their resistance to long periods of desiccation (Angel et al. 2011). Moreover, as PCR is based on
311 primers designed based on already known DNA sequences, it is possible that so far unrecognised methanogens
312 were left undetected. Thus, we cannot fully rule out the presence of an undetectably small, soil dwelling
313 methanogen population, which theoretically could produce some CH₄, which was then emitted from the non-
314 rooted soil and the shoots of *V. vitis-idaea*, *C. vulgaris*, and *P. sylvestris*.

315 Methanogens have also been suggested to colonise tree stems (Zeikus and Ward 1974; Mukhin and Voronin
316 2011), and thus an undetectable small methanogen population could as well have inhabited the stems of the
317 plants in our study. However, one reason why we did not detect methanogens in the plant material can be that
318 the plants were relatively young and grown in the laboratory, while wood decay and methanogens have
319 previously been found in stems of larger and older trees in the field (Berry and Beaton 1972; Zeikus and Ward
320 1974). In addition to transport of soil-derived CH₄ and the possibility of having undetected methanogens within
321 the plant material, the CH₄ emitted from the shoots may be produced in situ within the plants in aerobic abiotic
322 processes as suggested first by Keppler et al. (2006). Possible non-microbial origins of CH₄ are leaf surface
323 waxes, which have been detected to produce small amounts of CH₄ even without exposure to UV radiation
324 (Bruhn et al. 2014). Furthermore, saprotrophic fungi have also been identified as a source of CH₄ under aerobic
325 conditions (Lenhart et al. 2012). All the above mentioned mechanisms of CH₄ production have assumed to be
326 possibly common within plants.

327 Mature *P. sylvestris* trees have been demonstrated to emit small amounts of CH₄ under field conditions
328 (Machacova et al. 2016). The CH₄ emissions from *P. sylvestris* stems and shoots in a boreal upland forest under
329 field conditions were 0.31 and 3.1 nmol h⁻¹ m⁻² (medians), respectively (Machacova et al., 2016). If we convert
330 these emissions to emissions per plant biomass, using the biomass of the needles calculated by Machacova et
331 al. (2016) (with the function by Repola et al. (2007)) (4.4 kg), and the projected needle area (10–31 m² per tree)
332 (Machacova et al. 2016), the median flux from the needles under field condition is 0.017 nmol h⁻¹ g⁻¹.
333 Furthermore, if we calculate the total biomass of the branches including needles with an equation by Marklund
334 (1988), by using the mean tree height and the mean stem diameter at breast height from Machacova et al.
335 (2016), we get a larger total biomass (14 kg), and thus the median flux of 0.005 nmol h⁻¹ g⁻¹ from the branches.
336 In the microcosm experiment, we measured CH₄ fluxes between –2.7 and 3.3 nmol h⁻¹ g⁻¹ from the shoots of *P.*
337 *sylvestris*. These fluxes cover the range observed in the field, but indicate much higher CH₄ exchange rates. This
338 is in line with Pangala et al. (2015), who demonstrated that young trees can have significantly greater stem CH₄

emissions than mature trees. Part of the disparity results from upscaling of leaf-level CH₄ emissions measured in the field to the whole-tree level, since both the biomass functions and the projected needle area include certain assumptions and thus they are only estimates, and also from the exclusion of the stems from the upscaling.

The net fluxes of the seedlings suggest that for most of the species the shoot emissions contribute substantially to the net CH₄ exchange of these plant-soil systems (median contribution 91%). Even though the net fluxes of the microcosms are not directly comparable to the natural conditions, as in our experimental setup the above- and belowground compartments were separated, our results indicate that the ground vegetation is affecting the flux measured e.g. with the soil chambers and it should be taken into account in the experimental designs and result interpretations. The CH₄ uptake by the soils in the microcosms was not necessarily as strong as it is in boreal forest soils naturally. At the site where the soil and the seeds for the microcosms were collected the net fluxes of the boreal forest floor have shown strong CH₄ uptake based on the soil chamber measurements (Skiba et al. 2009; Machacova et al. 2016). In our experiment there was a relatively small amount of homogenised humus soil in each microcosm, and thus the disturbance and the lack of the natural soil horizons has presumably reduced the CH₄ oxidation capacity in the soil. It is also noteworthy that the qPCR method does not give information about the activity of the detected methanotrophs.

The results of the flux measurements and the qPCR indicate that the plant roots increase the CH₄ uptake by the methanotrophs in humus soils. The amounts of methanotrophs were mainly in line with the belowground fluxes: *V. vitis-idaea* soils had the most methanotrophs and the strongest average CH₄ uptake, while the non-rooted soil had significantly less methanotrophs than the ericoid shrubs and seemed to be a small source of CH₄. Praeg et al. (2016) also reported increased CH₄ uptake by forest soils with tree roots compared to non-rooted forest soil. The differences between the rooted and non-rooted soils are related to the presence of roots, which are in interdependent relationship with the soil microbes via the root exudates (Broeckling et al. 2008). Alternatively, it is possible that the non-rooted soils were wetter than the rooted soils, as there was no plant evaporation, which may have created more suitable conditions for microbial CH₄ production. The results also show some variation in both the belowground CH₄ fluxes and the amount of methanotrophs between the species, *V. vitis-idaea*-rooted-soils indicating the strongest uptake. The effect of plant species and vegetation types on soil CH₄ fluxes has also been shown by other studies (Praeg et al. 2016; Maier et al. 2017b). These differences can result from the different plant species effects on soil microbes, which may again result from the root exudates (Innes et al. 2004).

Upland forest soils typically favour methanotrophs specialized in oxidizing CH₄ at atmospheric mixing ratio (Kolb 2009 and the references therein). As the primer mb661r did not work well with our samples and the primer A650r did, most of the methanotrophs in our samples likely belong to these so called high-affinity methanotrophs covered by the A650r and not by mb661r (Bourne et al. 2001). The numbers of the methanotrophs match the qPCR results from other forest soil studies (Kolb et al. 2005; Knief et al. 2006).

The same microcosms were used for soil characteristic (Adamczyk et al. 2016) and bacterial amount analyses (Timonen et al. 2016), and the relationships between these variables and the CH₄ fluxes were also investigated. In our experiment, the soil pH was on average 4.5 in the non-rooted soils and 3.8 in the rooted soils (measured by Adamczyk et al. 2016). The *C. vulgaris* soils had the lowest pH, on average 3.7, and within *C. vulgaris* soils the number of methanotrophs was the second highest. The non-rooted soils were less populated by the methanotrophs and had higher pH compared to the rooted soils. Also the total amounts of bacteria were lower in the non-rooted soils than the rooted soils (Timonen et al. 2016), indicating that roots support the bacteria in the soil and lower the soil pH to more suitable level for the bacteria. The lower pH is probably also more suitable for the boreal soil methanotrophs, even though the correlation analysis did not reveal any correlation between the soil pH and the fluxes. In addition, the heterotrophic bacterial diversity has shown to increase the

CH₄ oxidation (Ho et al. 2014), which is in line with our observation that the increase in the amount of bacteria in the roots increases the CH₄ oxidation in the rooted soils.

The boreal forest soil methanotrophs are probably well adapted to low soil N content, because N is usually the limiting nutrient in these environments. This is supported by the findings that many methanotrophs are able to fix atmospheric N₂ (Murrell and Dalton 1983; Auman et al. 2001; Dam et al. 2012). We found that an increase in NH₄ concentration (measured by Adamczyk et al. 2016) increases the CH₄ uptake in the rooted soils, which is in line with previous findings that in small amounts increasing available N stimulates CH₄ uptake (Aronson and Helliker 2010), even though in larger amounts and during long time periods N fertilisation can inhibit CH₄ oxidation (Steudler et al. 1989; Mosier et al. 1991; Gullledge et al. 2004). In case of the non-rooted soils the correlation between the NH₄ concentration and the CH₄ flux was not significant (Online Resource 1) probably due to less methanotrophs in the non-rooted soils, although the NH₄ concentration was much higher in the non-rooted than in the rooted soils (Adamczyk et al. 2016). Overall the biogeochemical interactions between the soil N and CH₄ fluxes are complex and require more research.

Humus soil of the boreal forests contains high concentrations of phenolic compounds (Smolander et al. 2012 and the references therein). Phenolic compounds have been shown to inhibit microbial activity, including methanogens (Olguin-Lora et al. 2003; Wang et al. 2015; Poirier et al. 2016), and inhibition of pure cultures of methanotrophs has also been reported (Amaral and Knowles 1997). Our results are somewhat conflicting showing that the more the water-soluble phenols in soil the more CH₄ uptake in case of *P. sylvestris*, but an increase in the water-soluble phenols or the condensed tannins (measured by Adamczyk et al. 2016) in the soil cause an increase in the CH₄ emissions from the shoots of all the studied species. Based on our results it is difficult to draw solid conclusions about the possible effects of phenols on CH₄ fluxes.

The results of our experiment indicate that the aboveground parts of common boreal dwarf shrubs and *P. sylvestris* are able to emit CH₄. As our objective was to assess the roles of above- and belowground parts to the CH₄ fluxes, the results demonstrate that vegetation has an important and previously unrecognized role in the CH₄ flux dynamics of boreal plant-soil systems. The aim of this study was also to evaluate the contribution of methanogens and methanotrophs to the CH₄ exchange of common shrubs and *P. sylvestris* seedlings. The amount of methanotrophs in the rooted soil samples was mostly in line with the CH₄ uptake of the belowground compartments of the microcosms indicating that the plant roots enhance the methanotrophic activity and thus the soil uptake of CH₄. Although we did not detect methanogens in the shoots, roots or soil, we cannot exclude the possibility of methanogenic contribution to the net CH₄ flux from the studied plants. Our findings from this microcosm experiment demonstrate that more research should be directed to understand the interactions between plants, soil, and the microbial community driving the CH₄ exchange.

Acknowledgements

This study has been supported by the Emil Aaltonen Foundation, The Academy of Finland Centre of Excellence (project 272041), Academy of Finland Research Fellow projects (292699, 263858, 288494), InGOS, ICOS (271878), ICOS-Finland (281255) and ICOS-ERIC (281250) funded by the Academy of Finland.

Conflict of Interest: The authors declare that they have no conflict of interest.

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597

Plant and Soil

Above- and belowground fluxes of methane from boreal dwarf shrubs and *Pinus sylvestris* seedlings

Table 1. The arithmetic means $\pm$ standard errors of the means, medians, minimum and maximum of the CH₄ fluxes in nmol h⁻¹ and nmol h⁻¹ g⁻¹ (DW of the total plant and/or soil mass), and the number of microcosms in each group. The statistics of the fluxes are given for the whole plant-soil system (net flux), and separately for the belowground (belowg.) and aboveground (aboveg.) compartments.

	CH ₄ Flux (nmol h ⁻¹)					CH ₄ Flux (nmol h ⁻¹ g ⁻¹)					n
	Mean ± SE	Median	Min	Max	Mean ± SE	Median	Min	Max			
<i>V. myrtillus</i> (net flux)	-0.14 ± 1.0	-0.45	-3.9	3.9	-0.0029 ± 0.028	-0.012	-0.11	0.11	8		
Belowg.	-0.025 ^a ± 0.32	0	-1.2	1.7	-0.0070 ^a ± 0.0091	0	-0.035	0.049	8		
Aboveg.	-0.16 ± 0.93	0	-3.9	3.9	-0.045 ± 0.61	0	-3.2	3.0	8		
<i>V. vitis-idaea</i> (net flux)	-1.7 ± 0.46	-2.1	-3.5	0.044	-0.044 ± 0.012	-0.052	-0.086	0.0012	8		
Belowg.	-1.2 ^{b*} ± 0.41	-1.2	-2.8	0	-0.035 ^{b*} ± 0.012	-0.036	-0.081	0	8		
Aboveg.	-0.46 ± 0.55	0	-3.5	1.7	-0.028 ± 0.15	0	-0.71	0.83	8		
<i>C. vulgaris</i> (net flux)	3.2 ± 1.4	3.8	-2.8	7.7	0.084 ± 0.036	0.10	-0.078	0.20	8		
Belowg.	-0.051 ^a ± 0.24	0	-1.5	0.75	-0.0015 ^a ± 0.0071	0	-0.044	0.022	8		
Aboveg.	3.2 ± 1.5	4.2	-3.6	7.3	1.1 ± 0.64	1.2	-2.0	3.2	8		
<i>P. sylvestris</i> (net flux)	1.2 ± 1.5	0.78	-7.2	6.3	0.032 ± 0.041	0.021	-0.20	0.17	8		
Belowg.	-0.35 ^{ab} ± 0.16	-0.19	-1.2	0	-0.010 ^{ab} ± 0.0045	-0.0054	-0.035	0	8		
Aboveg.	1.6 ± 1.5	0.97	-6.6	6.7	0.54 ± 0.61	0.41	-2.7	3.3	8		
Non-rooted Soil	0.59 ^a ± 0.37	0.44	-1.1	3.5	0.019 ^a ± 0.013	0.011	-0.039	0.12	11		

^{ab} Means of the belowground fluxes denoted by a different letter are significantly different ($p < 0.05$)

* Significant difference from zero ($p < 0.05$).

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