Science of the Total Environment 586 (2017) 142–151

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

CH_4 and CO_2 production below two contrasting peatland micro-relief forms: An inhibitor and $\delta^{13}C$ study

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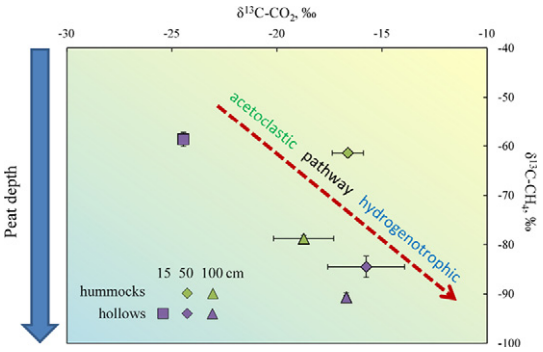
HIGHLIGHTS

- Micro-relief significantly affected CH_4 but not CO_2 production in surface peat soil.
- Soil of 10–50 cm depth produced up to 90% of the CH_4 and 50% of the CO_2.
- Hollows' topsoil showed the highest relative contribution of acetoclastic pathway (92%).
- The contribution of hydrogenotrophic pathway of methanogenesis increased with depth.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 November 2016
Received in revised form 24 January 2017
Accepted 27 January 2017
Available online 3 February 2017

Editor: Jay Gan

Keywords:
Greenhouse gases
Boreal peatland
Micro-relief forms
Stable carbon isotope method
Methanogenic pathways
Methanogenesis inhibitor

ABSTRACT

Two peatland micro-relief forms (microforms) – hummocks and hollows – differ by their hydrological characteristics (water table level, i.e. oxic-anoxic conditions) and vegetation communities. We studied the CH_4 and CO_2 production potential and the localization of methanogenic pathways in both hummocks and hollows at depths of 15, 50, 100, 150 and 200 cm in a laboratory incubation experiment. For this purpose, we measured CH_4 and CO_2 production rates, peat elemental composition, as well as $\delta^{13}C$ values of gases and solids; the specific inhibitor of methanogenesis BES (2-bromo-ethane sulfonate, 1 mM) was aimed to preferentially block the acetoclastic pathway.

The cumulative CH_4 production of all depths was almost one fold higher in hollows than in hummocks, with no differences in CO_2. With depth, CO_2 and CH_4 production decreased, and the relative contribution of the hydrogenotrophic pathway of methanogenesis increased. The highest methanogenic activity among all depths and both microforms was measured at 15 cm of hollows (91%) at which the highest relative contribution of acetoclastic vs. hydrogenotrophic pathway (92 and 8%, respectively) was detected. For hummocks, the CH_4 production was the highest at 50 cm (82%), where relative contribution of acetoclastic methanogenesis comprised 89%. The addition of 1 mM BES was not selective and inhibited both methanogenic pathways in the soil. Thus,

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http://dx.doi.org/10.1016/j.scitotenv.2017.01.192

BES was less efficient in partitioning the pathways compared with the $\delta^{13}C$ signature. We conclude that the peat microforms – dry hummocks and wet hollows – play an important role for CH_4 but not for CO_2 production when the effects of living vegetation are excluded.

1. Introduction

Northern peatlands historically have been a sink for atmospheric carbon dioxide (CO_2). They also have the potential of releasing large amounts of CO_2 and methane (CH_4) into the atmosphere, both naturally or as a result of environmental and anthropogenic forcing (Limpens et al., 2008). Both CO_2 and CH_4 are important greenhouse gases (GHG, IPCC, 2014) whose balance in peatland ecosystems is regulated by multiple environmental factors. Among them are the water table level, which controls the aeration status of the peat (Moore and Knowles, 1989; Moore and Roulet, 1993; Granberg et al., 1997; Kettunen 2003), the peat quality, which reflects the decomposability of constituent substances (Svensson and Sundh 1992; Granberg et al., 1997; Yavitt et al., 2000), the vegetation, which regulates peat quality and the transfer of gases belowground and to the atmosphere (Whiting and Chanton, 1993; Bubier et al., 1995), and the temperature, which controls the microbial metabolic reactions (Crill et al., 1993; Granberg et al., 1997, 2001; Winden et al., 2012). In peatlands, pronounced changes in environmental conditions occur vertically with peat depth and horizontally via micro-relief. This highlights the role of the different locations, each with specific physical and biochemical conditions (Lai 2009). Micro-relief and peat depth determines the interaction between the atmosphere, vegetation and the subjacent peat (Sundh et al., 1994; Granberg et al., 1997; Bergman et al., 2000; Dorodnikov et al., 2011). Such interactions result in the formation of distinct micro-relief forms (microforms). Thus, depending on the surface elevation, three microforms are distinguished: elevated hummocks, depressed hollows and intermediate lawns (Bubier et al., 1993). Two contrasting microforms – hummocks and hollows – distinctly differ by the water table level, i.e. the subsurface of water-logged hollows is typically anaerobic as compared to drier hummocks, thereby stressing the difference in redox processes between the two microforms (Kettunen, 2003). Furthermore, the plant species composition is closely connected with the water table and moisture conditions (Waddington and Roulet, 1997). Vegetation controls the input of plant-derived deposits into the microforms, hence affecting the carbon turnover and the formation and emission of GHG (Ström et al., 2005). For example, in a boreal oligotrophic fen, the hollows-dominating *Scheuchzeria palustris* contributed 2–4 times more to methanogenesis than the hummocks-dominating *Eriophoprum vaginatum*. This difference was mainly caused by differences in rhizodeposition, i.e. the release of organic compounds by plant roots into their surrounding environment (Dorodnikov et al., 2011).

Most studies so far have focused on measuring aboveground GHG flux to the atmosphere as related to microform type. The CH_4 fluxes reportedly decrease in the order hollows > lawns > hummocks, and the highest fluxes from hollows were mainly explained by persistent water-logged conditions (Bubier et al., 1993; Granberg et al., 1997; Saarnio et al., 1997; Forbrich et al., 2010; Aleina et al., 2016). In contrast, the lower water table and higher soil temperatures were proposed as the main factors controlling CH_4 oxidation and CO_2 respiration rates in aerated hummocks (Granberg et al., 1997, Dalva et al., 2001; Becker et al., 2008; Gažovič et al., 2013). Similar to in situ measurements, incubation studies under controlled conditions show that the CH_4 production potential increases from hummocks through lawns to hollows (Saarnio et al., 1997; Juottonen et al., 2015; Robroek et al., 2015). Importantly, the available in vitro studies focused mostly on the top 20–30 cm (down to 1 m) peat profile, where the soil organic matter (SOM) decomposition rates and the vegetation effects are the highest. However, anaerobic deep peat layers (deeper than 1 m) produce and store enormous amounts of GHG and substantially contribute to the surface efflux (Waldron et al., 1999; Glaser et al., 2004; Clymo and Bryant 2008, Steinmann et al., 2008). Despite its importance, we still insufficiently understand the mechanisms controlling belowground CH_4 and CO_2 dynamics in profile layers deep below the subsurface of microforms.

Generally, CH_4 cycling in peatlands consists of CH_4 production (methanogenesis) in the anoxic parts of the soil by methanogenic archaea (methanogens) and CH_4 oxidation (methanotrophy) in presumably oxic layers (Lai, 2009). Methanogenesis involves two main pathways that can occur solitary or in parallel: (1) acetate cleavage (acetoclastic pathway), which mostly takes place in the presence of fresh soil SOM and (2) CO_2 reduction with hydrogen (H_2) (hydrogenotrophic pathway) when electron acceptors other than CO_2 are not available (Hornibrook et al., 1997; Popp et al., 1999). CO_2 production occurs during all respiratory pathways including anaerobic SOM fermentation and acetoclastic methanogenesis. In the oxic part of the soil it is also released by plant- and microbial respiration, together with methanotrophy. As described above, peatland microforms are distinguished by the thickness of the aerated zone in the peat and plant communities that supply microorganisms with organic substrates. This in turn may affect the proportion of the two methanogenesis types in hummocks and hollows, especially with depth (Dorodnikov et al., 2013). Interestingly, in contrast to the well-defined in situ pattern of an increasing contribution of hydrogenotrophic pathway to overall methanogenesis with depth (based on $\delta^{13}C$—CH_4 and on $\delta^{13}C$—CO_2 data, e.g. Hornibrook et al., 1997; Popp et al., 1999; Steinmann et al., 2008), microbial community studies report contradicting results. Thus, the main methanogenic microbial groups present in the upper peat layer (study-defined 10–40 cm) were identified as the hydrogen-utilizing methanogens – *Methanomicrobiales* (Galand et al., 2002, 2003). In contrast, in deep peat the dominant groups were related to *Methanocellales* (putative hydrogenotrophs, Liebner et al., 2015) and *Methanosarcinales*, which can perform both methanogenic pathways (Galand et al., 2002; Putkinen et al., 2009).

Among other factors controlling CO_2 and CH_4 production in peatlands, GHG fluxes could be altered by the deposition of compounds and availability of anions such as ammonium (NH_4^+), nitrate (NO_3^-), sulfate (SO_4^{2-}), metals, e.g. iron (Fe) (Granberg et al., 2001; Eriksson et al., 2010; Sutton-Grier et al., 2011; Lozanovska et al., 2016). Peatlands are supplied with N and S compounds mainly through anthropogenic eutrophication of inland waters and/or acidic deposition from the atmosphere (Sutton-Grier et al., 2011). Along with the nutrition effect of N, S and Fe compounds for the plant- and microbial communities, these elements participate in redox reactions as alternative electron acceptors when oxygen availability is low. The presence of alternative electron acceptors can reduce CH_4 production due to a combination of inhibition and competitive effects between methanogens and other microorganisms for electron donors (Bodegom and Stams, 1999; Eriksson et al., 2010).

Under laboratory conditions, the mechanisms involved in CH_4 and related CO_2 dynamics can be studied using an approach with a specific inhibitor of methanogenesis, 2-bromo-ethane sulfonate (BES). BES is known to inhibit the reductive demethylation of methyl-Coenzyme M (Müller et al., 1993), a coenzyme required for methanogenesis. BES added at a certain concentration reportedly inhibits the acetoclastic – but not the hydrogenotrophic – pathway of CH_4 production (Zinder et al., 1984). Therefore, amending peat soil with BES may help to reveal

the distribution of methanogenic pathways between microforms and with depth. Another method to partition methanogenic pathways is based on the stable C isotope signatures (represented as $\delta^{13}C$ values) of CH_4 and CO_2, which reflect the CH_4 pathway formation (Whiticar, 1999; Conrad, 2005). Accordingly, CH_4 produced by the acetoclastic pathway is less ^{13}C depleted (e.g. shows higher $\delta^{13}C$ values) than CH_4 produced by the hydrogenotrophic pathway (lower $\delta^{13}C$ values). This is because methanogens more strongly discriminate against heavier ^{13}C during the latter process (Whiticar et al., 1986; Avery et al., 1999). The combination of both methods is assumed to provide strong evidence for the respective methanogenic pathway. If the inhibitor BES blocks CH_4 production by the acetoclastic pathway, then the respective $\delta^{13}C$–CH_4 signature should decrease due to a higher contribution of ^{13}C–depleted CH_4 produced by the hydrogenotrophic pathway as compared to the control (without inhibitor). Nonetheless, other important factors influencing $\delta^{13}C$ in CO_2 and CH_4, e.g. the $\delta^{13}C$ value of the organic substrate, fractionation during gas diffusion, CH_4 oxidation, must also be considered. Avery et al. (1999), Steinmann et al. (2008) and Clymo and Bryant (2008) gained valuable information about vertical and seasonal changes in the isotopic composition of CH_4 in peat profiles. Nonetheless, very little information is available about the effects of peatland micro-relief on the patterns of CH_4 and CO_2 isotopic signatures (Dorodnikov et al., 2013).

This study was designed to cover two aspects. Firstly, to estimate the production potential of CH_4 and CO_2 in the whole depth profile (down to 200 cm) below two contrasting microforms – wet hollows and dry hummocks – and to link this potential with the peat elemental composition. Secondly, to identify the contribution of the two methanogenic pathways in hummocks and hollows with depth by amending a specific inhibitor of methanogenesis and by measuring $\delta^{13}C$ in CH_4, CO_2 and peat organic matter. The following hypotheses were tested:

I. Under controlled anaerobic conditions, naturally wetter hollows will show an overall higher CH_4 but lower CO_2 production potential as compared with drier hummocks. This relationship should preferentially be caused by the adaptation mechanisms of microbial communities in microforms and be more pronounced in the upper peat layer.
II. With depth, the CH_4 and CO_2 production potential will decrease under both microforms, predominantly due to the decomposition state of the peat organic matter.
III. Due to higher availability of fresh plant-derived deposits in the upper vs. deeper peat layers, the contribution of the acetoclastic pathway to overall methanogenesis will decrease with depth below both microform types; between the microforms, more intensive rhizodeposition in hollows will promote the contribution of the acetoclastic pathway, as compared to hummocks, at least in the topsoil.

2. Materials and methods

2.1. Experimental site and peat soil collection

The experimental site is a central part of a natural minerogenic, oligotrophic low-sedge pine fen Salmisuo, located in the North Karelian Biosphere Reserve (62°47′N, 30°56′E) in eastern Finland. Detailed descriptions of the site are provided by several authors (Saarnio et al., 1997; Alm et al., 1999; Becker et al., 2008; Jager et al., 2009). The surface of the research area was subdivided into three main microforms in accordance with the topography, water table level and vegetation communities (Fig. 1): 1) elevated dry hummocks with an average water table during growing seasons between 15 and 20 cm below the peat surface and *Eriophorum vaginatum*, *Pinus sylvestris*, *Andromeda polifolia*, *Sphagnum fuscum* as dominant plant species, 2) intermediate lawns with an average water table from 5 to 15 cm below the peat surface and *Eriophorum vaginatum*, *Sphagnum balticum*, *Sphagnum papillosum* as dominant plant species, 3) depressed wet hollows with an average water table between 0 and 5 cm above the peat surface and the dominant plant species *Scheuchzeria palustris*, *Sphagnum balticum*, *Sphagnum majus* (Russow) C. Jens., *Sphagnum angustifolium* (Saarnio et al., 1997; Becker et al., 2008). Here, we tested the two contrasting microforms – hummocks and hollows. Peat samples were collected with a peat auger (Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands) – a stainless steel half-cylindrical sampler (50 cm long, 6 cm wide) with a massive cone and a cutting edge, sealed off by a hooked blade. Soil was sampled from both microforms and five depths: 15, 50, 100, 150 and 200 cm. Each true replicate consisted of a minimum of three randomly picked cores, of which a middle 10 cm section was collected and aggregated (e.g. to represent 15 cm depth level, a 10–20 cm peat layer was extracted).

2.2. CH_4, CO_2 production measurements and inhibition of methanogenesis

Peat soil samples from each of the five depths from both microforms ($n = 3$ true replicates) were aggregated in the laboratory and split into pseudo-replicates of 15 g fresh weight ($n = 5$ pseudo-replicates for each depth of hollows, $n = 6$ for each depth of hummocks). The resulting total number of 25 samples for hollows and 30 samples for hummocks were placed together with anaerobic indicator stripes (Microbiology Anaerotest, Merck, Darmstadt, Germany) in 150 ml Mason jars, which were closed by butyl rubber septa and screw caps. To create anaerobic conditions, the jars were connected to an evacuation line via needles with 3-way-stopcocks and flushed with pure N_2 for 20–30 min. After flushing, the jars were equilibrated to atmospheric pressure through a water lock and immediately filled with 15 ml pure N_2 to create overpressure to prevent air diffusion into the headspace and to enable subsequent sampling. The same procedure was repeated every time before gas production measurements.

To collect gas samples, a 60-ml gas-tight syringe was used to sample ca. 20 ml headspace gas, which was immediately transferred to a 12-ml pre-evacuated glass vial with overpressure. For each gas production measurement, four headspace gas samples were taken one after another at time intervals of 30–60 min after "zeroing" (headspace flush with N_2). The overall number of sampling days for each microform was eight, with the following chronological sequence (days): 0 (start of incubation and first sampling), 5, 9, 23, 37, 40, 43, 49 (end of incubation and last sampling of hollows) and 0, 15, 23, 37, 63, 70, 75, 79 (last sampling of hummocks). Thus, the sampling period for hollows was 49 days, for hummocks 79 days. This set-up reflected technical issues and the analytical routine of the laboratory. The gas production rates (see Section 3.1) at later incubation stages (after 40 days), however, did not differ significantly between microforms at a certain depth. Therefore, the lengthier duration of hummocks vs. hollows incubation had no pronounced effect on other parameters measured (isotope signatures, peat elemental composition, etc.).

The first four gas production measurements (days 0–23 for hollows and 0–37 for hummocks) served to determine the basal rates in all samples. At day 37 for hollows and at day 54 for hummocks, 1 mM of the inhibitor BES (pre-dissolved in 2 ml O_2-free milli-Q water) was added through the 3-way-stopcocks (without opening the jars) to three random jars of each depth, whereas the remaining jars (two of each depth for hollows and three of each depth for hummocks) served as controls with the addition of 2 ml O_2-free milli-Q water. The subsequent gas production was measured in the same manner as before addition of inhibitor and milli-Q water. Within the first days after BES amendment of hollows, no detectable difference in CH_4 production was observed; therefore, for hummocks, the GHG measurements started 9 days after adding BES.

The effective BES concentration was determined priorly in a testing experiment of CH_4 production from the same soil amended with 1, 10 and 100 mM of BES after Zinder et al. (1984) and Smemo and Yavitt (2007). The suppression of CH_4 formation with 1 mM concentration of

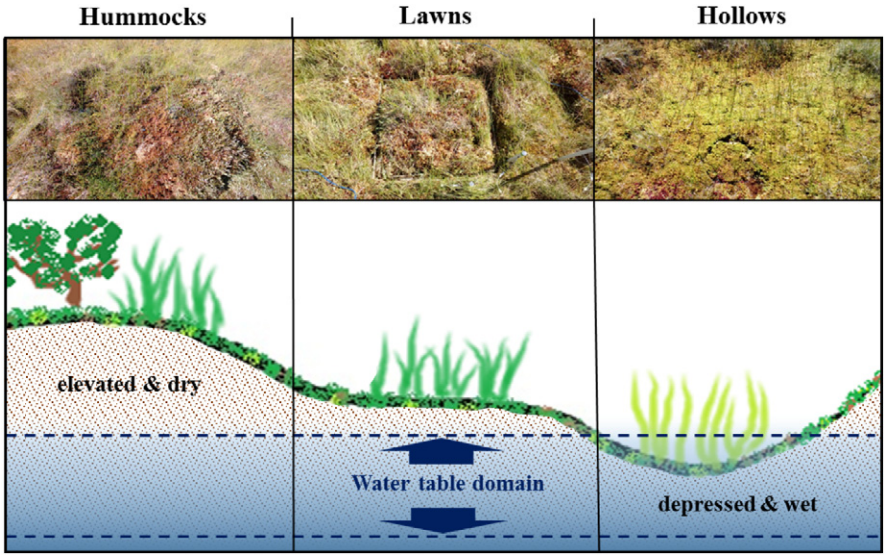

Fig. 1. Schematic view of a peatland topography demonstrating relief microforms: hummocks (elevated and dry), lawns (intermediate) and hollows (depressed and wet). Water table domain represents the approximate variation of the groundwater level during a growing season. The photos show examples of the microforms' vegetation cover at the study site (Photo: M. Dorodnikov).

BES was as effective as with 10 and 100 mM (data not shown). Thus, the lowest BES concentration was chosen in the main experiment.

During the experiment, all jars were stored at room temperature (about 22 °C) in the dark to avoid any possible production of oxygen by algae. CH_4 and CO_2 concentrations were measured on a gas chromatograph GC 6000 VEGASERIES 2 (Carlo Erba Instruments) equipped with a flame ionization detector, an electron capture detector and a pressure-controlled autosampler for 64 samples. Detailed information on the equipment can be found in Loftfield et al. (1997).

2.3. $\delta^{13}C$ analyses

To measure the stable C isotope composition in CO_2 (shown as $\delta^{13}C$–CO_2), a 1 ml headspace gas sample was taken as described above and diluted with pure N_2 in 1:60 ratio to obtain suitable concentrations for measurement on a cavity ring-down spectroscope (CRDS) Picarro G2131-i (Picarro, Inc., Santa Clara, CA, USA). The recommended minimum and maximum threshold CO_2 concentration of 380–2000 ppm was achieved. $\delta^{13}C$–CO_2 was measured for all samples ($n = 25$ for hollows and $n = 30$ for hummocks) three times during the incubation period for hollows (day 37 before adding BES or milli-Q water and days 49 and 79 after the addition as related to the start of incubation at day 0) and four times for hummocks (days 22 and 29 before addition and days 65 and 73 after addition).

Due to the requirements of the Isotope Ratio Mass Spectrometer (IRMS) for certain minimal CH_4 concentrations (<300 ppm), $\delta^{13}C$—CH_4 could only be measured in three soil layers (15, 50 and 100 cm) of both microforms shortly after the end of the incubation period. A headspace gas sample of 15 ml was taken as described above and transferred to a 12-ml pre-evacuated glass vial for $\delta^{13}C$—CH_4 measurement on a IRMS Delta C with a Conflo III interface (both from Thermo Fischer Scientific, Bremen, Germany) at the Centre for Stable Isotope Research and Analysis (KOSI), Büsgen-Institute, Georg August University Göttingen, Germany.

To measure $\delta^{13}C$ in solid samples, the peat soil was dried at 40 °C for several days, ball milled and weighed in tin caps. Samples were combusted in a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Cambridge, UK) and the $^{13}C/^{12}C$ ratio was measured on a Delta V Advantage IRMS with the Conflo III interface (Thermo Electron, Bremen, Germany) at KOSI shortly after the end of the incubation period.

2.4. Measurement of total extractable nitrogen, nitrate, ammonium, total sulfur, iron and pH

To measure dissolved total nitrogen (N_{extr}), nitrate (NO_3^-) and ammonium (NH_4^+), peat samples from both microforms and all depths were amended with milli-Q water in a proportion of 2:1 and shaken for 1.5 h. The obtained peat extracts were thoroughly filtrated several times: first, through a coarse paper filter (595 1/2, Whatman) into 50 ml centrifuge tubings. Then, to increase the output of solution from solid remnants, the latter were centrifuged at 2000 rpm for 5 min in containers with porous bottom and glass fiber filters. The extra solution was filtrated again through a paper filter and mixed with previously filtrated solution. The second filtration was done through fine syringe filters (Sartorius 0.20 μm pore size with luer lock, Göttingen, Germany) into 15-ml plastic centrifuge tubings. All filtrates were kept in a cold storage room at 4–6 °C prior to analysis. The concentrations of extractable N, NO_3^- and NH_4^+ were measured photometrically via Continuous-Flow-Analysis using multichannel peristaltic pumps (Cenco Instrumenten, Mij. N.V.Breda, Netherlands).

For the total sulfur (S) and iron (Fe) measurement, peat samples were dried (60 °C, 2–3 days) and ground to fine powder using a Fritsch Pulverisette (type 00.502, Oberstein, Germany) equipped with an agate pocket and ball mill. Total Fe and S content was then determined with an inductively coupled plasma (ICP) mass spectrometer (iCAP 6000 series, ASX-520 AutoSampler, Thermo Scientific, USA) after digestion of the samples in a mixture of nitric and hydrochloric acid (2:1 v:v) by a Digestore Milestone MLS 1200 (Microwave Laboratory System, Sorisole BG, Italy).

The pH values were measured with a pH-meter INGOLD (pH-electrode SenTix 21; Mettler Toledo). Replicates of each depth ($n = 2–3$) were dissolved in deionized water (DI-H_2O) in the proportion 1:2. The slurry was shaken for 1.5 h and pH measured directly in the slurry with an electrode. The obtained values ranged between 3.9 and 4.5 from 15 to 200 cm, respectively.

2.5. Calculations and statistical analysis

To calculate the gas production rate, four CH_4 and CO_2 concentrations (as ppb and ppm values, respectively) measured in each soil sample within 240–250 min were linearly approximated and the Ideal Gas

Law was used to convert the concentration from ppm/ppb to mass units per gram soil on a dry weight basis per hour (ng g d.w.$^{-1}$ h^{-1}). The BES effect was determined for each microform and depth by calculating the difference (in %) of the mean CH_4 production rate before and after adding BES. The difference was then corrected with respective control treatments by subtracting the effect of milli-Q water addition on the CH_4 production. The values were additionally "weighted" against each other according to their contribution to the cumulative CH_4 production. This was done by dividing the mean CH_4 production rate of each depth (e.g. 15 cm) by the sum of the mean CH_4 production rates of all depths (i.e. 15 cm + 50 cm + 100 cm + 150 cm + 200 cm) separately for each microform.

To estimate the contribution of each methanogenic pathway to total methanogenesis, an approach described by Conrad et al. (2005) and Angel et al. (2012) was applied. The approach is based on an isotope mixing model. For example, to calculate a relative fraction of hydrogenothrophic methanogenesis (f_{H2}) in the total produced CH_4, the following equation was used:

$$f_{H2} = (\delta_{CH4} - \delta_{ma})/(\delta_{mc} - \delta_{ma}) \quad (1)$$

where δCH_4 is the measured isotopic signature of C-CH_4, δ_{ma} and δ_{mc} are the specific isotopic signatures of the C-CH_4 produced solely by the acetoclastic and hydrogenotrophic methanogenesis, respectively. These specific isotopic signatures were estimated from the $\delta^{13}C$ values of SOM from each depth of two microforms based on stable carbon isotopic enrichment factors (ε) after Conrad et al. (2014). For hydrogenotrophic methanogenesis, $\varepsilon = -70‰$ and for acetoclastic methanogenesis, $\varepsilon = -31‰$ was used.

The required normality and homogeneity of the data were checked with the Shapiro-Wilk and the Levene's test, respectively. The variables were treated as independent for all depths below a microform and for a certain depth between microforms. The differences in CH_4, CO_2 productions and $\delta^{13}C$—CH_4, $\delta^{13}C$—CO_2, $\delta^{13}C$-peat values between microforms and depths were evaluated with two-way ANOVA and Tukey's HSD test. The CH_4 production rates before and after the addition of inhibitor BES were pairwise compared using paired *t*-tests. The significance of differences was determined at $P < 0.05$ level. All analyses were performed using R Statistical Software Version 3.2.3 (R Foundation for statistical computing).

3. Results

3.1. CH_4 and CO_2 production depending on microforms and depths

Based on the cumulative CH_4 production from all depths, hollows showed a significantly (almost one fold) higher CH_4 production rate than hummocks of ca. 22.4 ng CH_4 g d.w.$^{-1}$ h^{-1} (Table S1). The topsoil layer (15 cm) of hollows and the 50 cm layer of hummocks were significantly different from all other layers within each microform (Table S1a.). These two depths were also the main locations for CH_4 production, with a contribution of ca. 91% (41.7 ± 7 (mean ± SE) ng CH_4 g d.w.$^{-1}$ h^{-1}) and 82% (19.2 ± 2 ng CH_4 g d.w.$^{-1}$ h^{-1}) of the cumulative CH_4 production, respectively (Fig. 2a). These results agree well with the incubation studies of Bubier et al. (1993) and Saarnio et al. (1997) from the same peatland, and with Juottonen et al. (2015) on several peatlands in Finland and Estonia: all these studies reported generally higher CH_4 production rates in hollows vs. hummocks and the highest rates to be present in the 0–20 cm layers of hollows and 20–60 cm layers of hummocks.

At 50 cm depth of hollows, CH_4 production dropped to ca. 9% (3.9 ± 0.8 ng CH_4 g d.w.$^{-1}$ h^{-1}), whereas in hummocks at 15 cm the value was below 1% (0.3 ± 0.5 ng CH_4 g d.w.$^{-1}$ h^{-1}) of the overall CH_4 production. From 50 to 200 cm, CH_4 production substantially decreased to a minimum of <0.1 ng CH_4 g d.w.$^{-1}$ h^{-1} and there were no significant differences either between microforms or between depths.

CO_2 production did not differ between microforms at each soil layer, and the top soil layer of both microforms contributed 40–51% to the overall value (Fig. 2b). The production rate substantially decreased under both microforms by ca. 77% from the top (15 cm: 4153 ± 728 to 4997 ± 539 ng CO_2 g d.w.$^{-1}$ h^{-1}) to the deepest soil layer (200 cm: 923 ± 108 to 1216 ± 212 ng CO_2 g d.w.$^{-1}$ h^{-1}). A significant decrease was observed from the topsoil layer to the 50 cm layer (Table S1b). The contribution of deeper soil layers (50–200 cm) to the cumulative CO_2 production from all depths varied between 9 and 20% (Fig. 2b). The results agree with other reports of decreasing CO_2 production rates with depth (Moore and Dalva, 1997; Glatzel et al., 2004).

3.2. Effects of BES on CH_4 and CO_2 production

Those soil layers exhibiting the highest CH_4 production rates prior to adding the inhibitor BES – hollows 15, 50 cm and hummocks 50, 100 cm – showed a substantial decrease in CH_4 production rates after BES addition of −88%, −28%, −85% and −80%, respectively (Fig. 3). The decrease was statistically significant at 15 cm of hollows and 50, 100 cm of hummocks (Table S3). Remarkably, one control treatment in which only milli-Q water was added showed a decrease of CH_4 production similar to BES (hollows 15 cm, Fig. 3), although the decrease was less pronounced than in the BES treatments. After correction of the results (subtraction of the milli-Q water effect on CH_4 production decrease/increase in the respective control treatments) and "weighting" (depths below a microform against each other according to their contribution to cumulative CH_4 production), the suppressing effect of BES varied from 0 up to 68% (Fig. 4). Contrary to CH_4, the CO_2 production did not change after adding BES (data not shown).

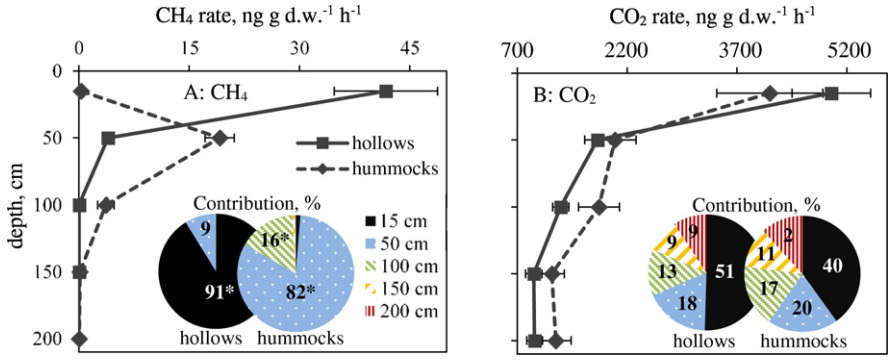

Fig. 2. Mean CH_4 (a) and CO_2 (b) production rate for hollows (continuous line) and hummocks (dashed line) at depths of 15, 50, 100, 150 and 200 cm without addition of the methanogenesis inhibitor (BES) or milli-Q water (samplings days 0–23 for hollows and 0–37 for hummocks). The integrated pie charts show the distribution (in %) of the overall CH_4 and CO_2 production from all depths. The contribution of depths below 100 cm to overall CH_4 production in hollows was <0.3%. In hummocks, depths 15, 150 and 200 cm comprised 1.3, 0.6 and 0.2% of cumulative CH_4, respectively. Asterisk: significant difference ($P < 0.05$) between the two microforms within the same depth.

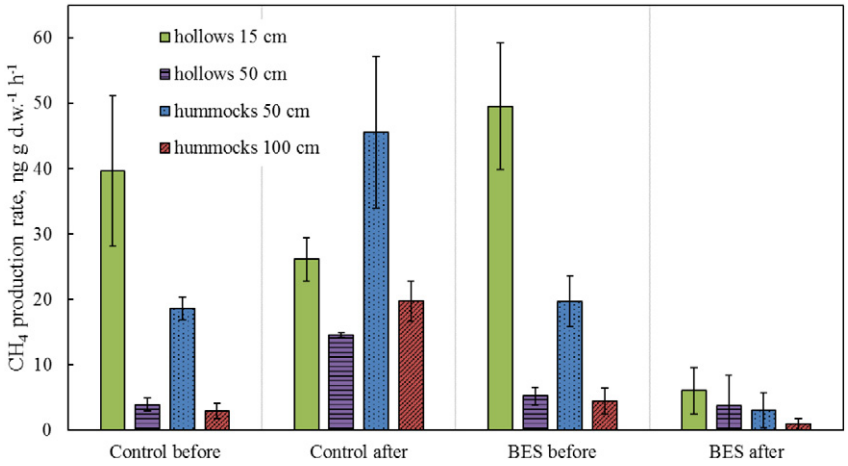

Fig. 3. Mean CH_4 production rates at two depth layers of hollows (15 and 50 cm) and two depth layers of hummocks (50 and 100 cm) before (sampling days 0–23 for hollows and 0–37 for hummocks) and after (sampling days 37–49 for hollows and 63–79 for hummocks) adding either milli-Q water (Control before, Control after) or methanogenic inhibitor BES dissolved in milli-Q water (BES before, BES after). The presented depth layers were the most productive and, accordingly, the drop in CH_4 production after BES addition was the most pronounced.

3.3. $\delta^{13}C$ of CO_2, CH_4 and soil organic matter

Generally, $\delta^{13}C$ of CO_2 varied substantially between depths, but the difference was less pronounced between microforms (Fig. 5a). CO_2 from the topsoil layer was the most depleted in ^{13}C ($\delta^{13}C$—CO_2 = −24.5 ± 1.5 (mean ± SE) ‰ for hollows and −29.3 ± 1.9‰ for hummocks, whereas at 50 cm depth CO_2 was the most enriched in ^{13}C (hummocks: −16.6 ± 0.5 and hollows: 16.7 ± 1‰) for both microforms. From 50 to 200 cm, a gradual depletion down to −20.6 ± 0.1‰ (hollows) and −23.6 ± 0.7‰ (hummocks) occurred. Among microforms, $\delta^{13}C$—CO_2 values were generally lower in hummocks than in hollows, although the pattern of $\delta^{13}C$—CO_2 change with depth was similar in both microforms.

In both microforms, $\delta^{13}C$—CH_4 values decreased with depth, ranging from −58.6 ± <0.1‰ at 15 cm to −90.7 ± 0.1‰ at 100 cm in hollows and from −61.4 ± 0.7‰ at 50 cm to −78.8 ± 1.4‰ at 100 cm in hummocks (Fig. 5b). The available data (for 50 and 100 cm) indicated significantly more depleted ^{13}C—CH_4 in hollows (Table S2).

Due to the limited information about the $\delta^{13}C$—CH_4, the calculation of the relative fraction of hydrogenothrophic methanogenesis was possible only for 15, 50, 100 cm depths of hollows and 50, 100 cm depths of hummocks. Both microforms showed an increasing trend with depth (Fig. 5b).

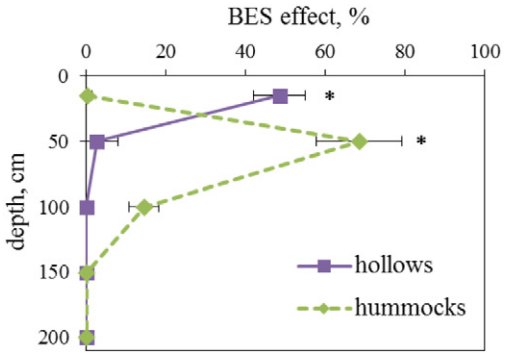

Fig. 4. BES suppression effect (in %) of the CH_4 production rate for hollows and hummocks down to 200 cm depth layer. The effect was calculated as the difference of the mean CH_4 production rate before (sampling days 0–23 for hollows and 0–37 for hummocks) and after (sampling days 37–49 for hollows and 63–79 for hummocks) adding BES. Changes in respective control treatments before and after adding milli-Q water were subtracted from the treatment effect. BES treatments of each microform were "weighted" against each depth according to their contribution to the cumulative CH_4 production of all depths. Asterisk: significant effects ($P < 0.05$).

The stable C composition of peat SOM ($\delta^{13}C$-peat values) was measured at 15, 50 and 200 cm (Fig. 5c). The values in the topsoil were higher in hollows (−24.5 ± 0.1‰) than in hummocks (−26.9 ± 0.1‰). At 50 cm, there was either a decrease (in hollows) or increase (in hummocks) to −26.3 ± <0.1‰ and −26.1 ± <0.1‰, respectively. In the deepest (200 cm) layer, $\delta^{13}C$-peat values further decreased (to −28 ± <0.1‰ in hollows and −27 ± <0.1‰ in hummocks).

3.4. Extractable N, NH_4^+, NO_3^-, S and Fe in soil

In general, both microforms showed an increasing trend of total extractable N (N_{extr}), NH_4^+, S and Fe concentrations with depth (Table 1). The measured NH_4^+ concentration was approximately half of N_{extr} within each depth and microform. The nitrate (NO_3^-) concentration was below the detection limit in all microforms and depths. Therefore, the difference between total N_{extr} and NH_4^+ presumably corresponds to dissolved organic N (DON).

4. Discussion

4.1. CH_4 and CO_2 formation in microforms and with peat depth

4.1.1. CO_2 production potential

During the incubation period, the CO_2 production potential under anaerobic conditions was similar between hummocks and hollows at each of the depth layers (Fig. 2b). This finding contradicts the hypothesized lower CO_2 production from hummocks vs. hollows under anaerobic conditions due to the overall in situ lower water table level in the former (a lower water table level leads to better soil aeration and hence to a dominance of microbial communities that are better adapted to an O_2-rich environment than to anaerobic conditions). Similar non-significant differences in CO_2 production between hummocks and hollows, albeit under aerobic conditions, were reported for the same soil (Lozanovska et al., 2016). Our and Lozanovska's incubation studies contradict in situ measurements reporting >3-times-higher CO_2 emissions from hummocks compared with hollows (e.g. Becker et al., 2008). Such inconsistency may reflect either lower in situ soil respiration of hollows due to the higher water table level (decreased aeration), and/or an onsite higher contribution of root or rhizosphere respiration to the overall soil CO_2 flux (Kuzyakov, 2006) in hummocks. In contrast, under controlled conditions, the lack of the regulatory effect of microform-specific plant communities on native soil CO_2 flux resulted in similar CO_2 production rates. Another mechanism is related to the properties of soil microbial communities developing below microforms.

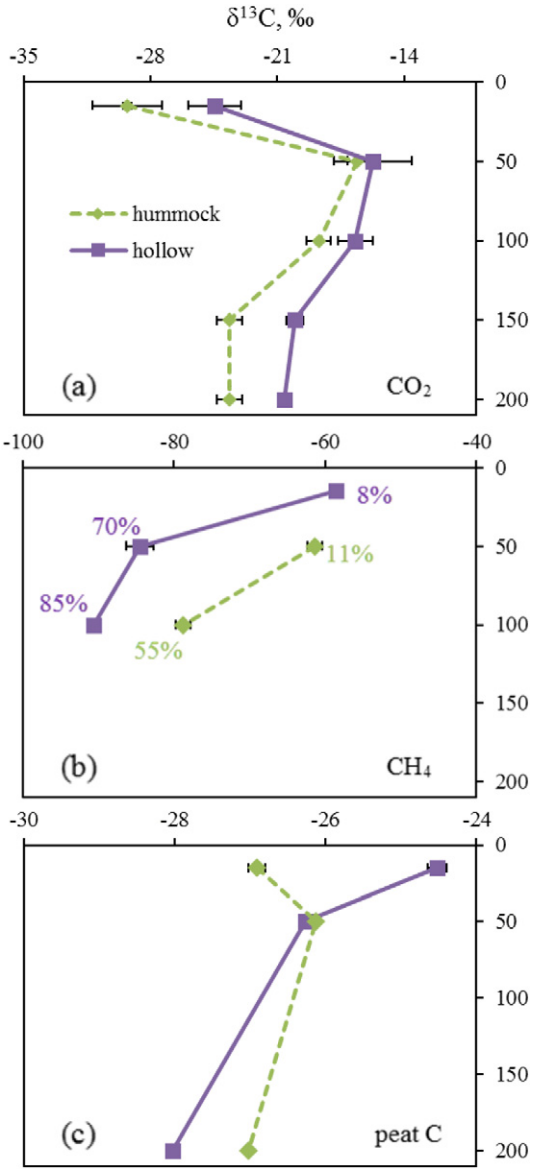

Fig. 5. Delta (δ) ^{13}C values of (a) CO_2 (n = 5–6 for each data point), (b) CH_4 (n = 3) with the relative fraction (in %) of hydrogenothrophic methanogenesis and (c) peat soil organic matter (n = 3) depending on depths of hollows and hummocks measured during the incubation period.

Table 1
Concentrations of extractable total nitrogen (N_{extr}, n = 2), ammonium (NH_4^+, n = 2) and total peat sulfur (S, n = 3) and iron (Fe, n = 3) for depth layers below hollows and hummocks (mean ± SE).

Microforms/depths	N_{extr} mg L^{-1}	NH_4^+	S mg g^{-1} d.w.	Fe
Hollows				
15 cm	0.21 ± 0.01	b.d.l.[a]	0.41 ± 0.01	0.31 ± 0.05
50 cm	1.72 ± 0.02	0.78 ± 0.03	1.15 ± 0.03	1.22 ± 0.09
100 cm	2.15 ± 0.01	0.94 ± 0.11	n.a.[b]	n.a.
150 cm	2.32 ± 0.07	1.18 ± 0.08	n.a.	n.a.
200 cm	3.08 ± 0.03	1.68 ± 0.02	1.57 ± 0.01	4.16 ± 0.12
Hummocks				
15 cm	b.d.l.	b.d.l.	0.45 ± 0.04	0.84 ± 0.12
50 cm	1.78 ± 0.03	0.91 ± 0.01	1.30 ± 0.01	1.14 ± 0.03
100 cm	2.28 ± 0.03	1.03 ± 0.01	n.a.	n.a.
150 cm	2.41 ± 0.14	1.32 ± 0.06	n.a.	n.a.
200 cm	2.55 ± 0.02	1.41 ± 0.04	1.43 ± 0.01	3.16 ± 0.05

[a] b.d.l. – concentrations below the detection limit of the measurement device.
[b] n.a. – not analyzed.

The naturally greater seasonal variations due to water table fluctuations in hummocks vs. permanently water-logged hollows promote the presence of aerobic and facultative anaerobic microbial species, supporting both aerobic and anaerobic SOM decomposition (Cord-Ruwisch et al., 1988). Therefore, incubation of hummocks´ topsoil under anaerobic conditions showed CO_2 production rates similar to those from the respective hollows samples. This interpretation must be tested by applying molecular methods to the microbial community structure in soils of the two microforms.

With depth, peat soil from both microforms showed decreasing rates of CO_2 production (Fig. 2b). This can be mainly explained by a decreasing availability of fresh SOM and by its degree of decomposition (Moore and Dalva, 1997; Glatzel et al., 2004).

4.1.2. Methanogenic potential

The hypothesized overall higher CH_4 production potential from hollows vs. hummocks (Fig. 2a) was confirmed. Dorodnikov et al. (2011) also found greater CH_4 production rates in peat soil from hollows compared with hummocks in a field labeling study of plant-soil cores from the same peatland. Thus, the hollows-dominating *Scheuchzeria palustris* contributed 2–4 times more to methanogenesis than the hummocks-dominating *Eriophoprum vaginatum*. This was mainly caused by differences in rhizodeposition, i.e. the release of organic compounds by plant roots into their surrounding environment. The trend of a decreasing CH_4 production rate from the topsoil layer to 100 cm depth (Fig. 2a) agrees with the hypothesized higher CH_4 production rates in upper vs. deeper layers. Similar to CO_2 production, this highlights the importance of specific depth-dependent biochemical and physical parameters, such as peat quality and nutrient availability, which influence microbial composition and activity, driving methanogenesis (Lai, 2009).

The topsoil of hollows was responsible for the highest CH_4 production (ca. 91%) among all depths, whereas the topsoil of hummocks surprisingly contributed <1% to overall CH_4 production (Fig. 2a). The in situ high O_2 availability in the hummocks' topsoil among other factors, controls the abundance of methanogenic microbial groups. Oxygen acts as an inhibitor or toxic agent for strictly anaerobic microorganisms (Shen and Guiot, 1996). Hence, hummocks´ topsoil may not contain a sufficient amount of obligatory anaerobic methanogens, resulting in low CH_4 production even under controlled anaerobic conditions. Accordingly, Bergman et al. (2000) suggested that a variation within the active microbial biomass to be at least partly responsible for a high range of CH_4 production rates in hummocks. Moreover, Yavitt and Seidman-Zager (2006) suggested a greater frequency and duration of anaerobic conditions to be responsible for a larger active biomass of methanogens in hollows than in hummocks. According to another mechanism, methanogens could be outcompeted by microorganisms, which primarily perform more energetically favorable reactions with lower Free Gibbs Energy (ΔG) (Schink, 1997; Beer et al., 2008). Thus, reactions such as denitrification (Rubol et al., 2012; Schlesinger and Bernhart, 2013), SO_4^{2-} reduction (Lovley and Klug, 1983; Pester et al., 2012) or Fe transformation (Lovley et al., 1996; Cervantes et al., 2002) provide lower ΔG than methanogenesis when oxygen is not available. In the studied soil, evidence for the presence of such a mechanism was given by the information about macro- and microelements (Table 1). Their distribution showed a trend of increasing concentrations with peat depth, in contrast to the CH_4 production rates, which generally decreased with depth (Fig. 2a). For better understanding, we assumed that the dominating forms of Fe and S were Fe^{2+} and SO_4^{2-} ions in the studied soil, and we related the standard energy change of formation (Δ_fG^o) of CH_4 (rate-based) and the respective combined standard energy of the measured elements (Fig. S1). Although the correlations were weak, we observed a trend of increasing Δ_fG^o CH_4 with decreasing Δ_fG^o of the elements (approaching "0") in hollows but not in hummocks. Accordingly, we assume that the processes which drive methanogenesis in hummocks (especially in the topsoil) are dictated less by the availability of alternate electron acceptors rather than by

the lack (or low activity) of obligatory anaerobic methanogens. Nonetheless, as no other anaerobic processes except methanogenesis were followed in our study, the mentioned mechanism should be tested in additional experiments by measuring the anion and cation concentrations as well as gaseous products (e.g. N_2O for nitrification/denitrification).

4.2. Estimation of methanogenic pathways based on $\delta^{13}C$ of CH_4, CO_2 and inhibition by BES

The use of specific inhibitors in combination with stable isotopes is a reliable method for the determination of CH_4 sources (Conrad, 2005). Among inhibitors for methanogenesis, 2-bromo-ethane sulfonate (BES) at a concentration of 1 mM was proposed to distinguish between two pathways – hydrogenotrophic (CO_2 reduction with H_2) and acetoclastic (acetate splitting) (Zinder et al., 1984). BES generally inhibited CH_4 production in the two microforms and at all depths (Fig. 4). Unfortunately, very low CH_4 concentrations hindered the $\delta^{13}C$—CH_4 analyses in samples with BES. Thus, the hypothesis about partitioning between methanogenic pathways could not be rigorously proven. Nonetheless, the $\delta^{13}C$ of CH_4 and CO_2 of the control confirmed the hypothesis of the decreasing contribution of the acetoclastic pathway of methanogenesis with depth and the increasing contribution of the hydrogenotrophic pathway. This finding corroborates earlier in situ measurements (Dorodnikov et al., 2013) by eliminating uncertainties evolved through the effects of diffusion and oxidation on $\delta^{13}C$ signatures in the field, especially in the top 1 m depth of microforms. Advantageously, controlled conditions excluded aerobic CH_4 oxidation and the substantial isotope fractionation during diffusion, which both exerted up to 70% bias of the acetoclastic methanogenesis signal in $\delta^{13}C$—CH_4 measured in situ (Dorodnikov et al., 2013). Since the suppression of CH_4 production with BES was substantial in all samples, even when the hydrogenotrophic pathway dominated before the addition, its inhibition was not selective, i.e. both hydrogenotrophic and acetoclastic pathways were blocked. Importantly, the reported concentration (1 mM) was tested in pure cultures of microorganisms and thus it may vary for complex natural objects such as soils.

In the current study, ^{13}C—CH_4 depletion with depth (Fig. 5b) reflected an increasing contribution of the hydrogenotrophic pathway to overall methanogenesis with $\delta^{13}C$—CH_4 values between −65 and −50‰ that typically correspond to the acetoclastic methanogenesis (Whiticar et al., 1986). Noteworthy, the most intensive CH_4 production took place in the hollows topsoil (Fig. 2a), where the most ^{13}C enriched CH_4 (−59‰) was detected (Fig. 5b). Simultaneously, the released $\delta^{13}C$—CO_2 was close to the $\delta^{13}C$ value of the native peat organic matter (ca. −24‰, Fig. 5a, c). This indicated both the restricted CH_4 oxidation (in this case $\delta^{13}C$—CO_2 should be closer to the $\delta^{13}C$—CH_4 source) and relatively low contribution of the hydrogenotrophic pathway to methanogenesis. In this case, ^{13}C—CO_2 becomes more enriched due to the preference of the methanogens for $^{12}CO_2$ against the heavier $^{13}CO_2$ (Popp et al., 1999). Lower $\delta^{13}C$—CO_2 values in hummocks vs. hollows in the top layer (Fig. 5a) reflected the small difference in C isotopic characteristics between the respective peat-SOM of the two microforms (Fig. 5c). This, in turn, is connected with the $\delta^{13}C$ signature of initial plant residues because different species dominated the two microforms (Becker et al., 2008; Dorodnikov et al., 2011). Therefore, we conclude that methanogenesis in the topsoil of hollows, as hypothesized, was dominated by the acetoclastic pathway (Fig. 6). This conclusion is additionally supported by the calculated very low (8%) relative fraction of hydrogenothrophic methanogenesis in the total produced CH_4 (Fig. 5b). The combination of on-site and off-site studies on methanogenic pathways are crucial to overcome the uncertainties resulting from the naturally occurring CH_4 diffusion and oxidation on the $\delta^{13}C$ signature, which may strongly mask the acetoclastic pathway in the upper peat layers.

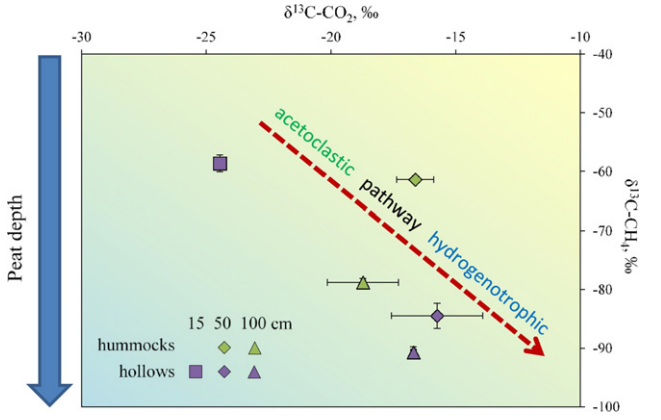

Fig. 6. Cross-plot of $\delta^{13}C$ of CH_4 and CO_2 (± SE) demonstrating the shift in methanogenesis (red dashed arrow) from the acetoclastic to the hydrogenotrophic pathway with peat depth below hummocks (green) and hollows (purple). Depths where both parameters were measurable are shown (15, 50 and 100 cm). Background color reflects the gradient in $\delta^{13}C$ from the lowest (blueish, left bottom corner) to the highest (yellowish, upper right corner) values.

In contrast to the topsoil, the $\delta^{13}C$ values of SOM at 50 cm depth were similar for both microforms, but the released CO_2 was ca. 10‰ more enriched than SOM (Fig. 5a, c). As described above, the CO_2 enrichment occurs during the hydrogenotrophic pathway of methanogenesis (Popp et al., 1999). However, $\delta^{13}C$—CH_4 at 50 cm was ca. 23‰ higher in hummocks than in hollows (Fig. 5b), indicating that the acetoclastic and hydrogenotrophic pathways may co-exist. This finding was additionally supported by the calculated relative fraction of hydrogenothrophic methanogenesis in the total produced CH_4 – 70% for hollows and 11% for hummocks (Fig. 5b) – and partly corroborates the results on the depth-dependent distribution of methanogenic microbial communities at the same peatland (Galand et al., 2002). Thus, below 40 cm, the contribution of *Methanosarcinales* – methanogens conducting both CH_4 production pathways – to total microbial community was increased, supporting potentially co-existing methanogenic pathways. Nonetheless, the information on the microbial community in general, and on methanogenic community structure specifically, should be considered with caution. The same study (Galand et al., 2002) reported the dominance of *Methanomicrobiales* – hydrogenotrophic methanogens – in the upper 10–40 cm peat layer. This contradicts the isotope-based evidence for the increased contribution of the acetoclastic pathway in the topsoil of peatland obtained here and in numerous other studies (Hornibrook et al., 1997; Popp et al., 1999; Chasar et al., 2000). Note also that other recent findings suggest that hydrogenotrophic methanogenesis is related to the occurrence of *Methanocellales* – putative hydrogenotrophs (Liebner et al., 2015). The apparent discrepancy between the molecular analyses of microbial community structure and the microbial ecology may reflect the nature of both approaches, when information on the availability of specific microbial groups is uncoupled from their functional performance. Therefore, future studies on GHG turnover in various ecosystems should combine molecular techniques (e.g. RNA) with labeling (e.g. ^{13}C, ^{14}C, ^{15}N) to reveal the metabolic pathways and activity of individual microbial groups.

5. Conclusions

CH_4 and CO_2 production and their $\delta^{13}C$ signatures before and after BES addition in soil from below two contrasting microforms – dry hummocks and wet hollows – revealed that: (i) CH_4 production was higher in hollows than in hummocks, but CO_2 production was similar between microforms (Hypothesis I conditionally supported); (ii) CH_4 and CO_2 production was higher in the surface peat compared to deeper layers

(Hypothesis II supported); (iii) the overall higher contribution of acetoclastic vs. hydrogenotrophic methanogenesis was valid for the upper peat layer of hollows; however, due to low methanogenic potential in the upper peat of hummocks, the comparison of methanogenic pathways with hollows was not possible (Hypothesis III conditionally supported). Inhibition with BES was less efficient in partitioning the two methanogenic pathways compared with the partitioning based on $\delta^{13}C$ signature. We conclude that the peat microforms – dry hummocks and wet hollows – play an important role for CH_4 but not for the CO_2 production when the effects of living vegetation are excluded.

Outlook

The study showed that micro-relief forms are important for the GHG balance. They should be considered as complex objects with unique combinations of environmental conditions such as water table level, plant communities and microbial populations. Therefore, predictions and modeling of GHG emissions in peatlands should consider the micro-relief and the mechanisms of CH_4 production.

Acknowledgements

Authors are thankful to technical staff of the Department of Soil Science of Temperate Ecosystems - Anita Kriegel, the Department of Agricultural Science - Ingrid Ostermeyer, Karin Schmidt, Susann Enzmann and the Centre for Stable Isotope Research and Analysis (KOSI) - Dr. Jens Dyckmans and Reinhard Langel from the Georg-August University of Göttingen for invaluable help in laboratory work and instrumental measurements. Authors would also like to thank four anonymous reviewers and the Editor (Dr. Jay Gan) for valuable suggestions and recommendations on the manuscript improvement. The study was sponsored by German Research Foundation (Deutsche Forschungsgemeinschaft, DFG). Project numbers DFG DO 1533/1-1 and DFG DO 1533/2-1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.01.192.

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