

Belowground plant traits and hydrology control microbiome composition and methane flux in temperate fen mesocosms

Marc Piecha
Jürgen Kreyling
John Couvenberg
Michael Pester
Anke Günther
Gerald Jurasinski
Gesche Blume-Werry
Tim Urich
Haitao Wang (✉ haitao.wang@uni-greifswald.de)

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Abstract

The rewetting of formerly drained peatlands is a strategy to fight against global warming through the reduction of CO₂ emissions, although this can lead to elevated CH₄ emissions. The interplay between plants, hydrology and microbiomes as ultimate determinants of CH₄ dynamics is still poorly understood, despite recent progress in field studies.

Using a mesocosm approach, we simulated the re-cultivation of a degraded temperate fen with three different water levels and two different plant over the course of a growing season. Peat samples for microbiome analysis, above- and below-ground plant biomass and gas fluxes were measured in April, June, August and October. Microbiome composition in top and subsoils was determined using 16S rRNA gene amplicon sequencing.

We found that peat depth and sampling time were the major factors shaping the microbiome composition dynamics. While plant species had a less strong impact, the difference to bare ground microbiomes was significant, especially in the lower layer. The water status also affected the microbiome, albeit to a much lesser extent. Methanogens were most abundant in the deeper peat and also more abundant in bare ground and *Carex rostrata* pots, as compared to *Juncus inflexus* or mixed pots. This was inversely linked to the larger root network size of *J. inflexus*. The methane emissions correlated positively with the abundance of methanogens and correlated negatively with the root network size. Despite the absence and low abundance of methanotrophs in many samples, the structural equation model suggested that the methanogen and methanotroph abundances together determined CH₄ fluxes.

In conclusion, this interdisciplinary study sheds light on how the complex interplay between plants, hydrology and the fen microbiome affect CH₄ emissions. It showed that the presence of plants as well as the plant functional type determine the abundance of methanogens and microbiome composition and thereby the resulting CH₄ fluxes accordingly.

1 Introduction

Peatlands cover an area of approximately 4.2 million km², which only amounts for 2.84 % of total earth surface (Xu et al., 2018), yet storing up to 24 % of earth total terrestrial organic carbon (OC, Zauft et al., 2010). Peatlands produce significant quantities of the powerful greenhouse gas (GHG) methane (CH₄, Günther et al., 2020). The degradation and drainage of peatlands for peat extraction and for agriculture, oxygenates the normally anoxic peat and, due to increased aerobic OC mineralization processes by the peat microbiome, results in massive emissions of the GHG CO₂ to the atmosphere (Joosten & Couwenberg, 2009). Renaturing and rewetting degraded peatlands are promising nature-based technologies to decrease these CO₂ emissions, to turn these peatlands again into sinks of CO₂. The process of rewetting, however, led to significant changes in local (plant) species that differ greatly from original peatlands, thus forming novel ecosystems, whose impacts on the peat microbiome are not well understood yet (Emsens et al., 2020; Kreyling et al., 2021). Since water content is a major driving factor

for changes in pro- and eukaryotic communities, rewetting does also change the GHG fluxes (Weil et al., 2020). Especially an increase in CH₄ emissions due to higher activity of methane-producing archaea (methanogens) might be a potential problem (Putkinen et al., 2018; Reumer et al., 2018; Urbanová & Bárta, 2020; Wen et al., 2018).

The interplay between plants, hydrology and the peat microbiomes as ultimate determinants of CH₄ dynamics is still poorly understood, despite recent progress in field studies. Investigating all the environmental effects on the microbiome, especially on the methane producing archaea, and the resulting changes in the GHG (e.g. Methane) emissions, is in fact challenging. For instance, water table fluctuations and nutrient dynamics as well as parameters such as salinity, nitrogen and phosphorus concentrations are influenced by seasonal dynamics (Feng et al., 2020; Kieckbusch & Schrautzer, 2007; Wang et al., 2021). Additionally, plants and their roots are in close interaction with the peat soil microbiome, henceforth changes in vegetation also affects the microbiome composition (Elliott et al., 2015; Ritson et al., 2021; Ward et al., 2015). The aforementioned studies have shown that plants have an impact on the soil microbiome and that the microbiome itself is the major source of methane. The relationship between those two kingdoms has been broadly studied (Elliott et al., 2015; Ritson et al., 2021; Ward et al., 2015), but only a few studies have aimed to assess all of them.

However, due to the complexity, *in situ* studies struggle to provide explicit linkages between plants, hydrology, microbiome composition and activity, and ultimately robust explanatory models for CH₄ fluxes. In consequence, mesocosm studies might well be suited to resolve some of these linkages and might be a good compromise between reduced complexity and higher experimental control as compared to *in situ* studies.

Using such a mesocosm approach, we aimed to simulate the re-cultivation of a degraded temperate fen by using deep peat soil cores from a percolation fen in northern Germany. We simulated three different water level dynamics as well as colonization with two different indigenous plants, *Carex rostrata* and *Juncus inflexus* and a mixture of both during one growing season. With such a design we were able to investigate the development of a deep peat microbiome under the impact of those factors. Furthermore, tight measurement of GHG emissions, plant- and root biomass changes gave us the opportunity to dissect the relative importance of those factors on the microbiome dynamics and ultimately CH₄ fluxes. We hypothesized that hydrology, i.e. water table manipulations had a larger effect than plant identity and composition on the microbiome composition and resulting CH₄ fluxes and that microbiome composition, especially methanogen abundance are tightly linked to CH₄ fluxes.

2 Material and methods

2.1 Experimental design

The mesocosm study was conducted as part of WETSCAPES (wetscapes.de) in order to research the effects of rewetting on peat soils. For this purpose, 2 m deep peat was excavated out of a percolation fen

near the village Tribsees (northern Germany) in 2018. The peat was evenly mixed and distributed into 36 plant pots (3 water status × 4 plant communities × 3 replicates) which were then inoculated with ditch water nearby the sampling location (Fig. 1). Three water status were assigned to simulate different peatland conditions: stable high for natural sites, stable low for drained sites and fluctuating for rewetted sites. Furthermore, in autumn of 2018, indigenous plant species, *Carex rostrata*, *Juncus inflexus* and their mixture were introduced to the pots, pots without any plants were considered as the control. We had a consistent control on the weight of pots and the height of the water level, and a continuous measurement of greenhouse gas (GHG) fluxes, root and aboveground biomass and pore-water characteristics (DOC, NH_4^+ , NO_3^- and NO_2^-).

2.2 Root network size and CH_4 flux

Root network size was monitored using minirhizotrons as described in Schwieger et al. (2022). Briefly, one transparent tube was installed in each pot at an angle of 45° in the soil to insert a root image scanner (CI-600 In-Situ Root Imager; CID Bio-science Inc.), taking c. 350° scans (image size: 21.6 × 19.6 cm) of the tube-soil interface and thus roots at two depths (0-5 and 25-30 cm). Light was excluded from the tubes with a cap and the tubes were wrapped with mirror foil to reduce thermal differences. The root network was scanned biweekly, and the images collected were further analyzed according to Schwieger et al. (2022).

The CH_4 flux was measured similarly as in the field according to Wang et al. (2023). During the measurement, an opaque circular chamber made from polyurethane was fixed on each pot for 3 min. The chamber was equipped with fans to ensure the consistent mixing of the air inside during measurements. CH_4 concentrations were measured biweekly using either a GasScouter (Picarro, Santa Clara) or an Ultra-Portable Greenhouse Gas Analyzer (Los Gatos Research, Mountain). The calculation of the CH_4 flux was described in Wang et al. (2023).

2.3 Peat sampling and DNA extraction

We took triplicates from each mesocosm at four different time points in the year 2019 (April, June, August and October) from two different depths, upper peat layer (0-5 cm) and lower peat layer (25-30 cm). We mixed the corresponding triplicates on a sterile petri-dish and stored each peat sample in sterile Eppendorf-tube. We put those tubes immediately on dry ice and stored them at -70 °C until further processing. At each of the four time points, 72 samples (4 plant communities × 3 water levels × 2 depths × 3 replicates) were collected and ~250 mg material was subjected to DNA extraction using the Power Soil™ kit. The bead beating step was performed in a FastPrep® machine with the following parameters: intensity of 5 m/s for 45 s. The resulting DNA was quantified using Qubit™ 4 Fluorometer and stored at -80 °C.

2.3 Sequencing and data processing

Bacterial and archaeal 16S rRNA genes were amplified from the extracted DNA using a nested PCR protocol with the 16SF_515YF_lib and 16SR_B806R_lib primer pair (Apprill et al., 2015; Caporaso et al., 2011, 2018), targeting the V4 region of the 16S rRNA gene plus a sequence which was also used as the primer for the second round PCRs. The primers were also indexed for the second round PCRs. The amplicons were cleaned using the Nucleofast® 96 PCR-Clean-up kit (Macherey-Nagel), pooled and then sequenced using Illumina-MiSeq PE (2*250 bp) platform. After demultiplexing, the data was processed using the *dada2* protocol in R v4.0.3. The raw sequences were truncated at 250 and 200 bps for forward and reverse reads, respectively. Sequences failing to meet the filter criteria (maxEE = 2, truncQ = 2, maxN = 0) were discarded. Those filtered sequences were de-replicated, the amplicon-sequencing-variants (ASVs) were deduced, and the paired-end sequences were merged. Afterwards the chimeric sequences were removed. The sequence of each ASV was assigned to taxonomy using the SILVA SSUref_NR_138 database. Furthermore, ASVs with only one sequence and ASVs that were assigned to mitochondria and chloroplasts were removed. Additionally, samples with sequence numbers below 3000 were removed, leaving 256 samples for downstream analysis, with a total of 25329 ASVs.

2.4 Bioinformatics and statistics

The complete downstream analysis was done using R v4.0.3. Principle Coordinate Analysis (PCoA) based on the Bray-Curtis-Dissimilarity was performed to reveal the microbial community compositions. Furthermore, linear regressions were employed to depict correlations between relative abundance of methanogens and methanotrophs, methane fluxes and root network data. Pearson's correlations were used to test these correlations. The significance of the impact of factors on community compositions was determined by permutational multivariate analysis of variance (PERMANOVA) using *vegan* package v2.5.7. Kruskal-Wallis *post hoc* Dunn's tests were performed to compare the means of methanogen relative abundance, methane fluxes and root-network size between different vegetations, water status, depths, and time points using *vegan* package v2.5.7. The p-values were adjusted using the "false discovery rate" (fdr) method (Benjamini et al. 1995). In all cases the statistical significance level $\alpha = 0,05$ was used.

Structural equation modelling (SEM) was performed to find out the causes and consequences of microbiome changes by assuming that plants could directly influence CH₄ fluxes and indirectly through impacting the methanogen and methanotroph abundances which further determine CH₄ fluxes. SEM was constructed using covariance-based method with *lavaan* package (Rosseel 2012). All variables were checked for normality, and the non-normally distributed ones were log10 transformed. A minimum value was added to variables with negative values to generate positive values before log10 transformation, including methanogen and methanotroph relative abundance and their subtraction. The multivariate normality of the final dataset showed insignificant multivariate Kurtosis.

3. Results

3.1 Spatial and temporal dynamics of peat microbiome composition

The mesocosms experiment simulated one growing season of plants on restored fen peat, covering spring, summer and autumn seasons with samplings of microbiome, plant biomass and GHG fluxes (Fig. 1). Using 16S rRNA gene amplicon sequencing, we obtained microbiome (bacteria and archaea) compositions. Using multivariate analyses, we investigated the spatial and temporal dynamics in the microbiomes at two depths across the growth period. The PCoA-plot (Fig. 2A) showed that location in the peat had the strongest impact among all factors on the microbiomes (PERMANOVA $R^2 = 0.15$, $p = 0.001$), in which the upper peat layer microbiomes differed strongly from the ones in the lower layers. This, however, changed with time as the microbiomes developed a larger alpha-diversity in the upper layers (not shown), indicated by the variance. Especially the microbiomes were much more spread in August and October when compared with the microbiomes at the time points before. The impact of water status, vegetation and plant species for October can be seen in figure 2. For the water status (fig. 2B), there was only a minor difference between stable low and stable high, with the microbiomes from the fluctuating treatment located in between. This difference, however, was more prominent in the lower peat microbiomes than in the top peat microbiomes. For vegetation (fig 2C), there was a clear compositional difference between microbiomes from mesocosms with vegetation as compared to the bare peat pots. Especially in lower layer, the distinction was very clear. A detailed look into the plant species (fig. 2D) showed that there was a clear difference between the lower peat microbiomes in *Carex rostrata* and *Juncus inflexus* pots. Furthermore, microbiomes from the pots with both plant species were located between microbiomes from pots with both single species.

3.2 Methanogen and methanotroph compositions and abundances

To get insights into the functional guild responsible for CH_4 production, we analyzed composition and abundance of methanogenic archaea (Fig. 3) and methanotrophic bacteria (Fig. 4). The relative abundance of methanogens was rather low with less than 1% of all bacterial and archaeal 16S rRNA genes, as seen previously in peat soils (Jurasinski et al., 2020; Potter et al., 2017; Weil et al., 2020). Five orders of methanogenic archaea dominated were abundant, the *Methanosarciniales*, *Methanomicrobiales*, *Methanomassiliicoccales*, *Methanobacteriales* and *Methanocellales* (Fig 3A), indicating a diverse set of operational pathways, i.e. hydrogenotrophic, acetoclastic and methylotrophic methanogenesis (Conrad, 2019). At every time point, consistently higher relative abundances of methanogens were observed in the lower peat as compared to the top peat microbiomes (Fig. 3A). A decline of their relative abundance was observed with time in the plant pots, at both soil layers, while the abundance was rather stable in the bare peat (Fig. 3A). The constant decline of the methanogen abundance over time with plants resulted in a low abundance of methanogens in the plant pots compared to bare peat in October (Fig. 3B).

The relative abundance of methanotrophs was much lower, accounting for only up to 0.15% of the total microbiome (Fig. 4). The methanotroph abundance showed a similar change over time as observed with

methanogens in the plant pots, while the abundance in the bare peat declined in June and August, followed by a sharp increase in October (Fig. 4A). The methanotroph abundance was much higher in the top peat layer and in the bare peat (Fig. 4A and 4B), which may relate to oxygen availability and methanogen activity, respectively.

3.3 Links between microbiomes, plants, and methane fluxes

In order to understand the effect of different factors on methanogen abundance and composition, the relative abundance for methanogens in a later time point where a rather developed microbiome and plant cover existed is shown (Fig. 5). In October, the highest methanogen abundance was found with bare ground (~0.72%), followed by *C. rostrata* bare ground mesocosms (~0.14%). There was a significantly lower abundance of methanogens in pots with *J. inflexus* and with mixed species, compared with bare ground and *C. rostrata* pots (Fig. 5A). Similarly, methane fluxes were significantly higher in bare ground and *C. rostrata* pots than in *J. inflexus* and mixed species pots (Fig. 5B). However, the root network size showed a contrast pattern (Fig. 5C). Larger root networks were found in pots with *J. inflexus* and the mixed pots compared with bare and *C. rostrata* pots (Fig. 5C).

Linear correlation analysis revealed a significant, positive correlation between methanogen abundances and methane fluxes across all mesocosms (Fig. 6A). However, the root network size was significantly and negatively correlated with both methanogen abundance and CH₄ flux (Fig. 6B and 6C), suggesting a negative plant impact on methanogens and thus CH₄ fluxes in peat. Due to the low abundance and absence of methanotrophs in most samples, the correlations between methanotrophs and CH₄ fluxes could not be statistically verified. However, since methanogens and methanotrophs both impact CH₄ dynamics, the subtraction between methanogen and methanotroph abundances (MG-MT) was applied. Interestingly, an improved correlation with CH₄ fluxes was observed compared with methanogen abundance along (Fig. 6A and 6D).

A structural equation model was constructed to show the causes and consequences of microbiome changes in our mesocosm system (Fig. 6E). The concentration of dissolved organic carbon (DOC) was integrated in the model since DOC might be the major substrate for methanogenesis. The root network showed striking influences including a positive impact on methanotroph abundance and a negative impact on DOC, methanogen abundance and MG-MT. DOC showed no significant influence on the microbiomes, suggesting that substrates might not be a limiting factor in the peat mesocosms. Surprisingly, neither methanotroph nor methanogen abundance significantly impacted the CH₄ flux, while their subtraction showed a positive and strong causality on the CH₄ flux, suggesting that methanogens and methanotrophs together determined CH₄ fluxes in the studied ecosystem.

4 Discussion

The below-ground (micro-)biome and interactions therein are central to peatland C and N cycling (Robinson et al. 2023). Rewetting changes the peat microbiome (bacteria, archaea, fungi, protists; Weil et

al. 2020; Wang et al. 2021, 2022, 2023) as compared to drained fens (Emsens et al. 2020; Jurasinski et al. 2020; Kreyling et al. 2021). These shifts indicate drastically changed functions in the below-ground microbiome. For instance, the functional guild of methanogens within the complex peat microbiome is the most important determinant of the potential flux of CH₄ from wetlands to the atmosphere. To date, there is no complete understanding of the effects that the major biotic and abiotic drivers have onto the microbiome and the methanogenic archaea, respectively (Robinson et al. 2023).

Using a mesocosm approach, we assessed the effects of water level (abiotic factor) and plants (biotic factor) onto the microbiome composition and activity in a spatially-temporally resolved study design. We found a strong succession along during the time of the mesocosm experiment. This dynamic development of the original microbiome stemming from deep peat was also evident in the compositional difference between the microbiomes in the top and deeper peat layers at the onset of the sampling in April 2019. At that time the microbiomes that originally stemmed from the same deep peat sample had developed in the pots for six months.

While plant species identity had a less strong impact, the difference to bare ground microbiomes was significant, especially in the lower layer. The water status also affected the microbiome, albeit to a much lesser extent than expected. This contrasted our hypothesis that hydrology, i.e. water table level would have a large effect onto the microbiome composition. Such a minor effect was found also in another mesocosm experiment (Potter et al., 2017). This is in contrast to *in situ* studies, wherein the microbiomes of drained and rewetted sites differed strongly (Emsens et al., 2020; Weil et al., 2020). This controversy can have several reasons. For one, the microorganisms are resilient to short term changes in the water table and effects of drought and flooding (Andersen et al., 2013; Basiliko et al., 2013; Cadillo-Quiroz et al., 2020; Emsens et al., 2020; Ritson et al., 2021). Another explanation is that the deeper peat layers were always saturated with water during our experiment, thereof creating stable anoxic niches for microorganisms (Cadillo-Quiroz et al., 2020). Another reason might be related to the rather short time frame of our experiment, in which the microbiomes might not have had time to adapt (Cadillo-Quiroz et al., 2020), as compared to the decade-long rewetting in some of the *in situ* studies (Weil et al., 2020).

In contrast, we observed that in our study plant presence as well as plant identity played a more important role in the structuring of the microbiome, especially in the later time points of the growing season. This finding goes well in line with the view of plants as major architects and controllers of the belowground ecosystem. This was particularly evident when analyzing the consequences of plant presence for the methanogenic archaea and the resulting CH₄ fluxes. Methanogens were most abundant in the pots without vegetation, both in deep as well as the shallow peat microbiomes, while their abundance was lower in the presence of vegetation. Also, the abundance of methanogens kept declining over time as the plants grew and their root network expanded. One likely explanation for this effect was the development of a root network that facilitated the diffusion of oxygen into the peat, creating oxic and suboxic zones in an otherwise anoxic environment (Armstrong, 1967; Brune et al., 2000; Flessa & Fischer, 1992; Galand et al., 2005). Additionally, root exudates distributed through the roots to the surrounding

rhizosphere might have influenced the development of the microbiome as well (Badri et al., 2009; Chaparro et al., 2012; Galand et al., 2005).

It was striking to observe a differential effect of the plant species onto the methanogens and the general microbiome: mesocosms with *J. inflexus* had a lower abundance of methanogens as the ones with *C. rostrata*. The possible explanation of this effect is the belowground root network size, which was much larger for *J. inflexus*. A bigger root network likely distributes more oxygen in the surrounding area, which is detrimental for the obligatory anaerobic methanogens. This was evident when the methane emissions correlated positively with the abundance of methanogens but correlated negatively with the root network size.

While the methanogen abundance largely determined CH₄ fluxes, the integration of methanotroph abundance improved the quality of its correlation to CH₄ fluxes. It was surprising that only the subtraction between methanogen and methanotroph abundances significantly determined the CH₄ flux in the structural equation model, suggesting this relationship as a promising microbial proxy in predicting CH₄ flux in peat. Supporting this, the other study showed that the interaction between functional gene markers of methanogens and methanotrophs better correlated with CH₄ fluxes in response to summer drought in rewetted peatland soils, when compared to either marker alone (Wang et al., 2023). The next step is to establish a full predicting model by integrating the environmental and characteristics.

In conclusion, this interdisciplinary study sheds light on how the complex interplay between plants, hydrology and the fen microbiome affect CH₄ emissions. It showed that the presence of plants as well as the plant functional type determine the abundance of methanogens and methanotrophs, microbiome composition and thereby the resulting CH₄ fluxes accordingly. Thus, future restoration and rewetting projects including paludiculture, should take into consideration the plant species composition, if CH₄ emissions are an important factor for consideration.

Declarations

Conflict of interest

The authors declare no competing interests.

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Figures

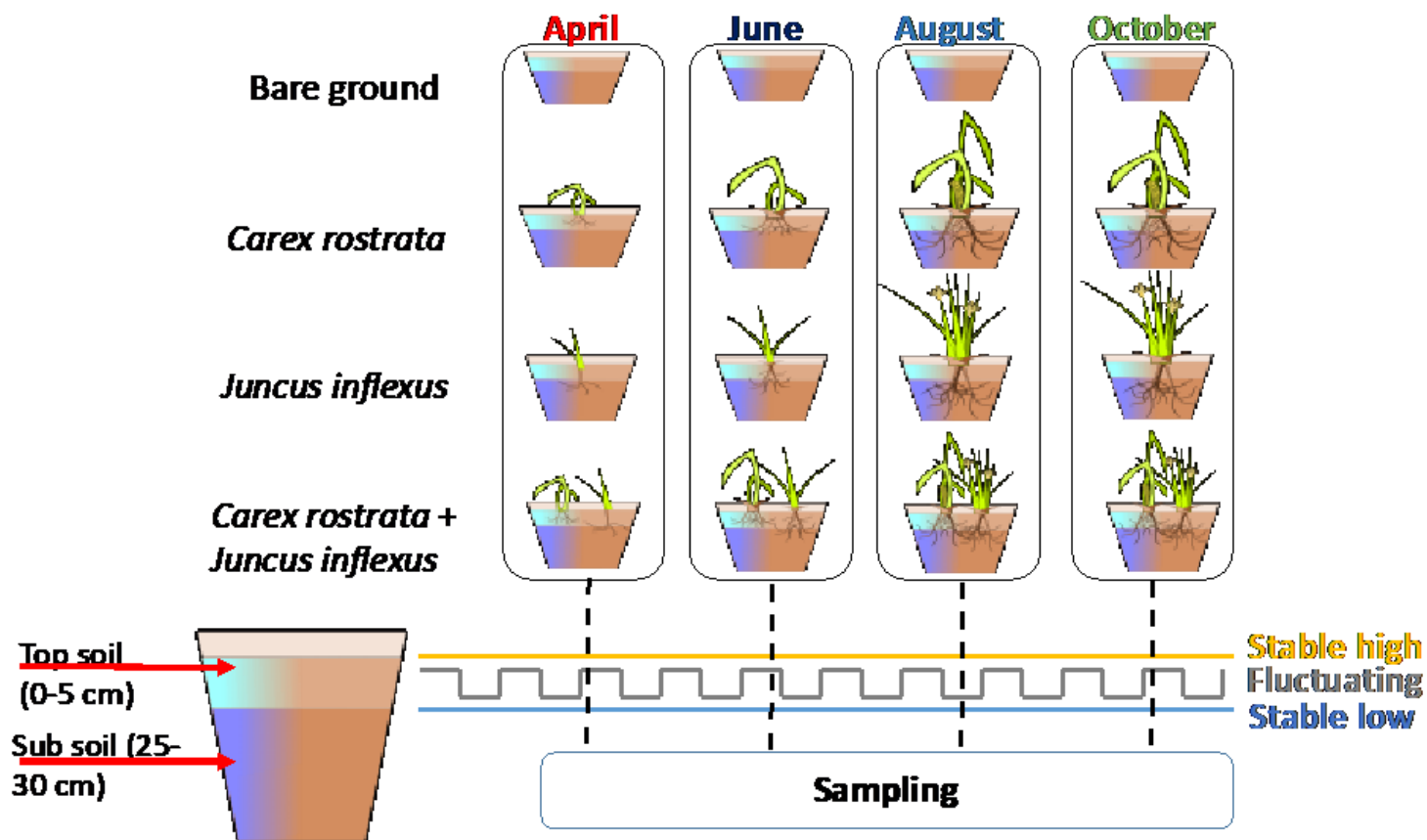


Figure 1

Scheme of the mesocosm experiment showing the pot setup with the corresponding water status, plant communities, sampling depth and sampling time points. The periods of fluctuating water level changed every two weeks.

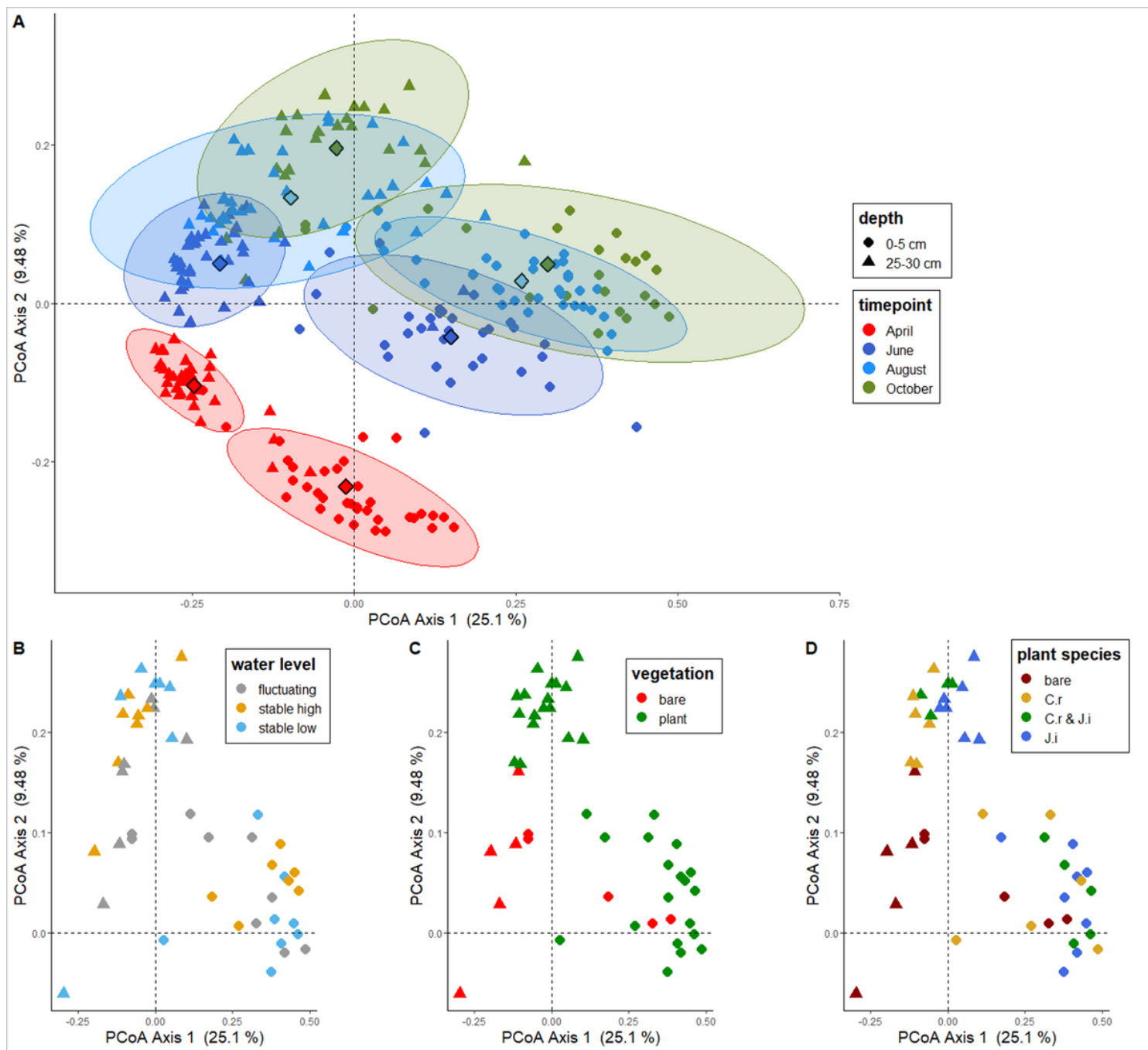


Figure 2

Spatial and temporal microbiome dynamics. A: PCoA plots of the peat microbiomes labelled by sampling time and depth. B: Microbiomes labelled by water level and depth (October). C: Microbiomes labelled by vegetation and depth (October). D: Microbiomes labelled by plant species and depth (October).

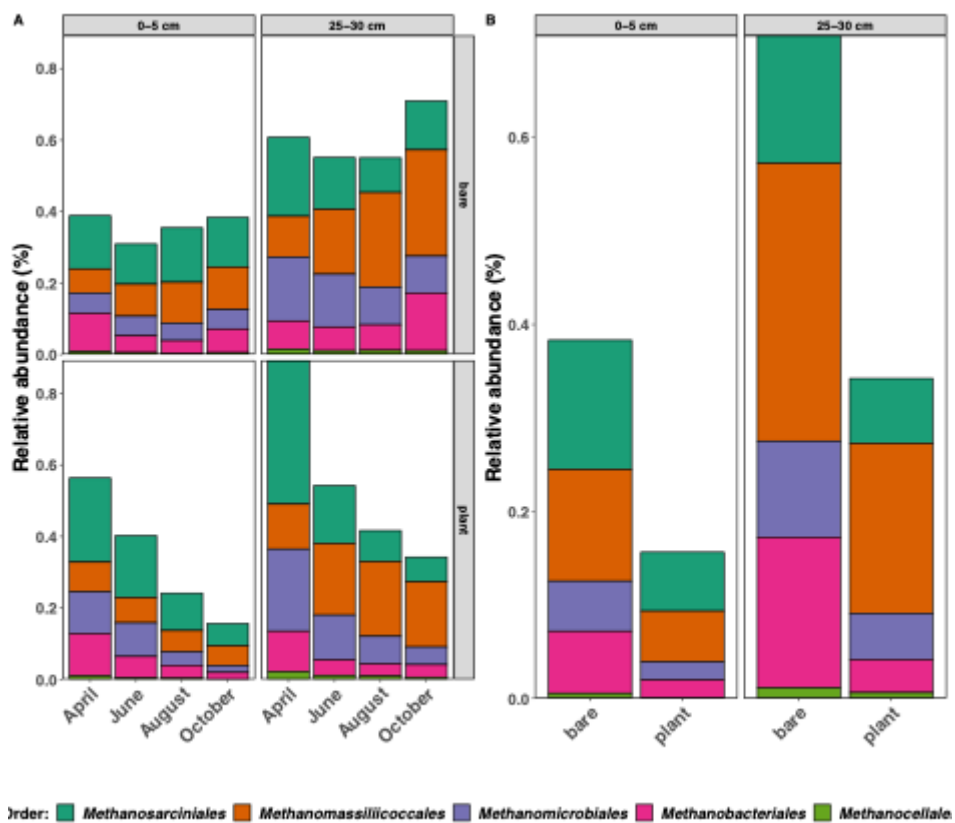


Figure 3

Barplots depicting the relative abundance of different methanogen orders for all time points (A) and October (B) at two depths. Bare, pots without any plants; plant, all pots with plants.

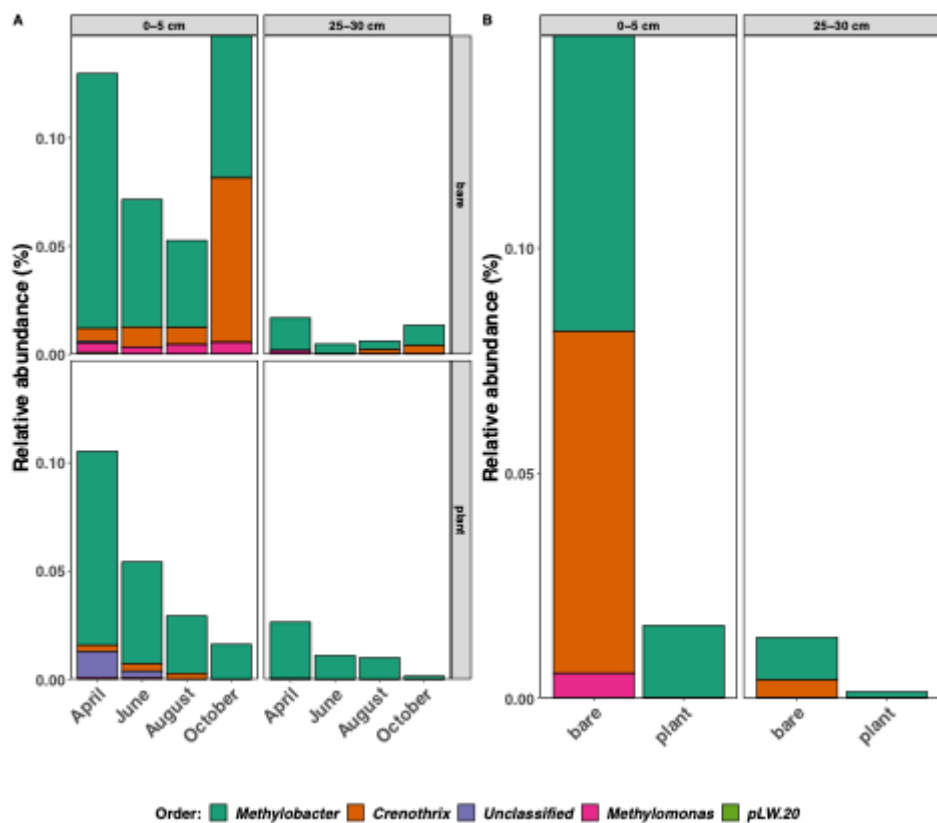


Figure 4

Barplots depicting the relative abundance of different methanotroph orders for all time points (A) and October (B) at two depths. Bare, pots without any plants; plant, all pots with plants.

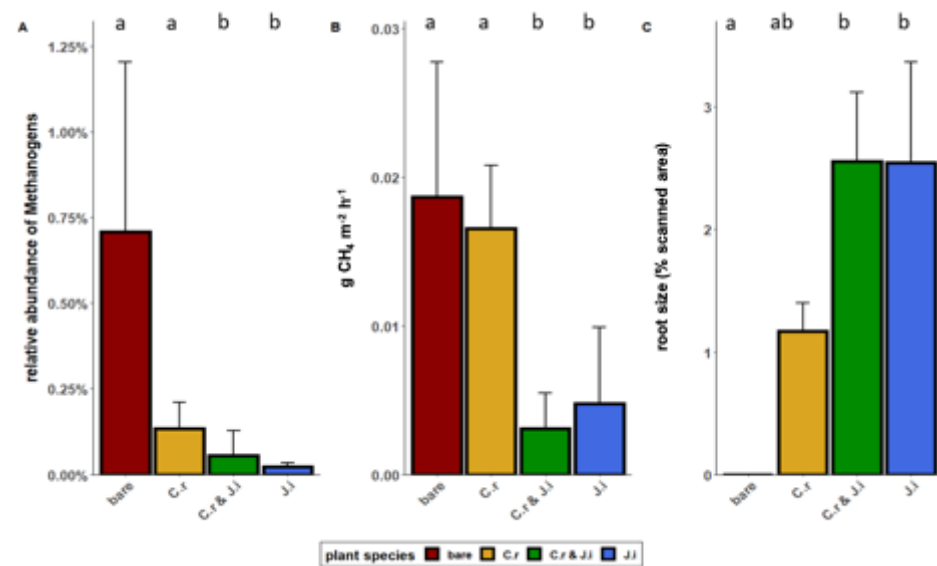


Figure 5

Barplots showing relative abundance of methanogens (A), methane flux ($\text{g CH}_4 \text{ m}^{-2} \text{ h}^{-1}$) (B) and root network size (% scanned area) (C) in bare ground and the corresponding plant community mesocosms in

October. Bars with “a” are significantly different to bars with “b” (adjusted $P < 0.05$).

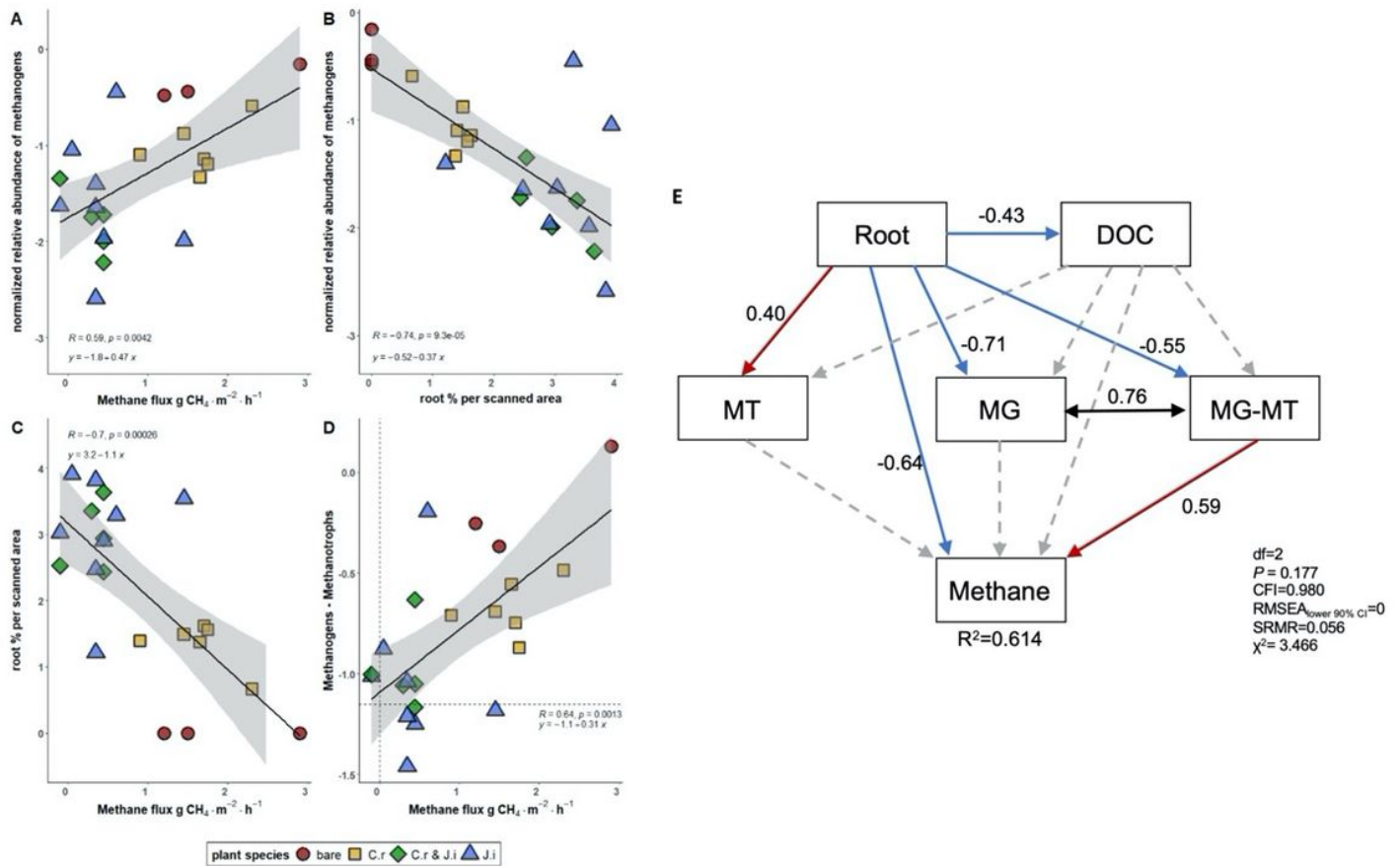


Figure 6

Correlation analyses between relative abundance of methanogens and methane flux (A), between relative abundance of methanogens and root size (B), between methanogens-methanotrophs abundance and methane flux (C), and structural equation model showing causes and consequences between different factors (D). The dotted lines indicate the 0 values in the original data. MG, methanogen abundance; MT, methanotroph abundance; MG-MT, subtraction between methanogen and methanotroph abundances.