

# Tree tissues and species traits modulate the microbial methane-cycling communities of the tree phyllosphere

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## Research Article

**Keywords:** methane, methanogens and methanotrophs, phyllosphere microbiota, tree-mediated methane emission, wetlands, floodplain, forested swamp

**Posted Date:** March 11th, 2025

**DOI:** <https://doi.org/10.21203/rs.3.rs-6066438/v1>

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**Additional Declarations:** No competing interests reported.

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# Abstract

## Background

Methanogenic and methanotrophic communities (i.e., the microbial communities involved in methane production and consumption) of the tree phyllosphere remain uncharacterized for most tree species despite increasing evidence of their role in regulating tree methane fluxes. Using 16S rRNA gene sequencing, we studied the methanogenic and methanotrophic communities of leaves, wood and bark of five tree species (*Acer saccharinum*, *Fraxinus nigra*, *Ulmus americana*, *Salix nigra*, and *Populus tremuloides*) growing in the floodplain of Lake St-Pierre (Québec).

## Results

Methanogenic and methanotrophic communities differed mostly between tree tissues (leaf, wood and bark) but also between tree species according to different traits (e.g., leaf, heartwood and bark pH, leaf and heartwood humidity). Methanogens were prevalent in the wood of trees, while facultative methanotrophs were found in higher proportions than methanogens in leaves and bark, suggesting different potential role of these microbial communities in methane regulation. Tree species differing in key traits could also be associated with differential microbial production/consumption of methane. Tissue pH was a particularly important trait in modulating methanogen-methanotroph community composition and the relative abundance of methanogens and methanotrophs in the different phyllosphere compartments.

## Conclusion

Our study shows that methanogens and methanotrophs are prevalent in the phyllosphere of several tree species, suggesting a potential widespread role in the regulation of tree methane fluxes. Tree species traits are important in determining the composition and abundance of phyllosphere methane-cycling microbial communities. Better understanding these microbial communities and their drivers can help assess their potential contribution to methane mitigation strategies.

## 1. Background

Soil methane ( $\text{CH}_4$ ) fluxes result from the balance between microbial production (i.e., methanogenesis) and consumption (i.e., methanotrophy). Forest soils  $\text{CH}_4$  budget tends toward net uptake due to conditions favorable to methanotrophy (e.g., good soil oxygenation) [1, 2]. In lowland forested ecosystems however, waterlogging creates anoxic conditions which favor methanogenesis over methanotrophy, resulting in net emissions [3]. It is now well known that trees can transport and release the  $\text{CH}_4$  produced in soils into the atmosphere, contributing to ecosystemic  $\text{CH}_4$  emissions [1] but trees can also consume  $\text{CH}_4$  and may therefore contribute to the global atmospheric  $\text{CH}_4$  uptake [4, 5]. The

dominance of methane-uptake over emissions, when considering the whole tree, [5] demonstrates the potential of trees as global atmospheric methane sinks.

Recent evidence provided by studies investigating microbial communities of the tree phyllosphere suggests that the tree microbiota may play a role in the regulation of tree methane fluxes. Notably, methanogens have been detected in the heartwood of poplars [6–8] and of mangrove tree species [9]. The role of heartwood methanogenic communities in CH<sub>4</sub> production and its regulation by wood humidity and secondary metabolites has been previously demonstrated [10, 11]. Methanotrophs have also been identified in wood but at lower relative abundances [6, 8]. Isotopic signatures of CH<sub>4</sub> have indicated that low-affinity methanotrophy at the stem base and high-affinity methanotrophy higher on the tree trunk would be responsible for net CH<sub>4</sub> uptake at the whole tree and stand levels under upland conditions [5].

Although less studied, methanogens and methanotrophs have also been identified in other tree compartments and could play a role in the regulation of tree methane fluxes. Jeffrey et al. [12] identified a methanotrophic community in the bark of *Melaleuca quinquenervia* which was responsible for the reduction of tree CH<sub>4</sub> emissions. In addition, leaf methane uptake, which suggests microbial oxidation of methane by methanotrophs, has been reported in previous studies [4, 13] and oxygen-tolerant methanogens as well as new monooxygenases potentially involved in CH<sub>4</sub> consumption have been identified in needles of Norway spruce [14]. Different niches for methanogens and methanotrophs exist within the tree phyllosphere due to heterogeneous physicochemical conditions prevailing in different tissues, notably in terms of oxygen availability, which is known to modulate these groups of microorganisms [5, 15]. Therefore, methane-cycling microbial communities are expected to differ among phyllosphere tissues and compartments.

Species traits can play an important role in structuring microbial communities within the tree phyllosphere [16]. Notably, for methane-cycling communities, traits which influence oxygen availability (e.g. wood density and humidity) can in turn influence the presence of methanogens and methanotrophs within the tree phyllosphere [17]. In addition, the nature and concentration of chemical compounds (e.g. carbohydrates, phenolic compounds, methanol, acetate) in the tissues and other traits such as vulnerability to rot can also influence the methane-cycling communities by modulating the availability of substrates [11, 17, 18]. Since methanogens and methanotrophs have different pH optimums and tolerances, the pH of tree tissues is another factor having the potential to structure these microbial communities [17, 19]. Methanogenesis is optimal at pH 7 and is reduced at lower pH, while methanotrophy is optimal at pH 5-6.5 and can be performed by acidophilic and acido-tolerant taxa at lower pH values [19, 20]. It is therefore expected that the methane-cycling communities of the tree phyllosphere will differ among species due to inter-specific variability in key traits. For instance, methanogens were detected in the heartwood of *Populus canadensis* but not in the heartwood of *Pinus tabulaeformis* in the study by Li et al. [11], a finding that was linked to differences in species traits.

Despite increasing evidence supporting the role of the tree microbiota in methane flux regulation, studies on methanogenic and methanotrophic communities of the tree phyllosphere remain limited to a small number of species and tissue types. Moreover, the relationship between methane-cycling communities and species traits remains poorly investigated. This study aims to characterize the phyllosphere methanogenic and methanotrophic communities of different tree species and assess the relationships with their traits. To do so, we collected leaf, wood and bark samples from five tree species (*Acer saccharinum*, *Fraxinus nigra*, *Ulmus americana*, *Salix nigra*, and *Populus tremuloides*) in the floodplain of Lake St-Pierre (Québec) and performed microbial community analyses to identify methanogens and methanotrophs within the tree phyllosphere. We then investigated how the methanogenic and methanotrophic communities of trees differ between tree tissues, compartments, species and according to several tree traits (wood and bark humidity, density and pH, stem diameter, leaf mass area, humidity and pH).

We hypothesized that:

- 1) Methanogenic and methanotrophic communities of the tree phyllosphere differ among tree tissues (leaf, wood, bark) and compartments (leaf epiphytes and endophytes, heartwood, sapwood, bark). More precisely, we hypothesized that methanogens have a higher relative abundance and alpha diversity in wood, where oxygen is limited, while methanotrophs have a higher relative abundance and alpha diversity in tissues where oxygen is available (i.e. leaves and bark).
- 2) Methanogenic and methanotrophic communities differ among tree species. Beyond the influence of tissues and compartments, we also consider that tree species offer contrasting habitats to methanogenic and methanotrophic communities.
- 3) The composition of methanogenic-methanotrophic communities is correlated to tree traits which regulate chemical conditions and oxygen availability (i.e., humidity and pH). More precisely, we hypothesized that the relative abundance of methanogens is positively correlated with tissue humidity and pH, while the relative abundance of methanotrophs is negatively correlated with tissue humidity and pH.

## 2. Material and Methods

### 2.1. Experimental Design

The study took place in the Lake St-Pierre floodplain in six blocks distributed on the south and north shore of the St-Lawrence River (Québec, Canada) (Additional file 1: Fig. S1). Each block was divided into two plots corresponding to locations exposed to high flood frequency (HFF) and low flood frequency (LFF) determined from historical records between 1970 and 2020 [21, 22]. In each plot, a mature tree with a DBH greater than 10 cm and no apparent sign of rot of the species *Acer saccharinum*, *Fraxinus nigra*, *Ulmus americana*, *Salix nigra* and *Populus tremuloides* (or *P. deltoides*) was selected for tissue sampling.

## 2.2. Tree tissue sampling and analysis

Sampling took place in the Summer of 2023 (July 18th to August 1st). Wood, bark, and leaf samples were collected for species traits and microbial analyses. Two wood cores and a bark sample (64 cm<sup>2</sup>) were collected at breast height using an auger and a wood knife, respectively, cleaning the equipment with ethanol between each tree. Leaves were collected at a height of 10 m using a pole and shear. Samples for DNA-based microbial community analyses were stored on ice in the field and frozen at -20°C once in the laboratory.

Each wood core was split into sapwood and heartwood based on coloration. Leaf, wood and bark samples for tree traits analyses were weighed on the day of sampling and dried at 65°C for 72h. The dry weight was then measured to calculate humidity, and the wood and bark volumes were assessed to calculate specific density. The leaf area was determined from a scan with ImageJ and the leaf mass per unit area (LMA) was calculated from the dry weight divided by the leaf area. Tissue pH was measured from fresh samples ground in liquid nitrogen and placed in solution in distilled water (1:5 M: V) and incubated for 1 hour with agitation.

The epiphytic community of leaves was collected using a washing protocol. A volume of 25 ml of autoclaved 1X phosphate buffer saline (PBS, Bio-Rad, Hercules, CA, USA) supplemented with 0.05% of Tween 20 (PBST) was added to the bags containing the leaf samples. Bags were installed on a rotary shaker at 300 rpm for 5 minutes. The leaves and the PBST were then recovered in a 50 ml tube and vortexed for 3 minutes at maximum speed. The leaves were removed from the tube and stored in a clean bag at -20°C until further processing for endophytic microbial community analyses. The PBST was centrifuged at 4000 g and 4°C for 20 minutes. The supernatant was removed using a pipet, leaving ~ 2 ml of PBST in which the pellet was resuspended and transferred into a 2 ml tube. An additional centrifugation step of 1 minute at 15 000 g was performed and the supernatant discarded. The pellet was resuspended in 800 µl of CD1 solution (QIAGEN DNeasy Powersoil Pro kit), transferred into a PowerBead tube and kept frozen until DNA extraction. For leaf endophytic community analysis, leaf samples washed with PBST were ground to a fine powder in liquid nitrogen using a mortar and pestle. Wood and bark samples were first ground using the electric Grinder Mill IKA A11, then with a mortar and pestle. A mass of 0.25g (wood and leaves) and 0.1g (bark) of the ground samples was added to the CD1 solution of the QIAGEN DNeasy Powersoil Pro extraction kit and stored at -20°C until DNA extraction.

## 2.3. DNA extraction and Illumina Sequencing

DNA was extracted with the QIAGEN DNeasy Powersoil Pro kit following the manufacturer's instructions and using the QIAcube instrument. DNA concentration was assessed using the Qubit dsDNA Quantification Assay kit (Invitrogen, Waltham, MA, USA). 16S rRNA gene library preparation was performed as described by Illumina [23] using user-defined primers (515F-Y, 5'-GTGYCAGCMGCCGCGGTAA and 926R, CCGYCAATTYMTTTRAGTTT – 3', ~ 412bp amplicon) [24] with some modifications. Peptide nucleic acid (PNA) PCR blockers (PNA Bio Inc., Thousand Oaks, CA, USA) were included in the PCR reaction to inhibit the amplification of plant chloroplast and mitochondrial DNA

[25]. The PCR reaction included 25 µl HotStarTaq plus MasterMix (Qiagen), 0.5 µl of the forward and reverse primers (10 µM), 2.5 µl of mPNA and pPNA PCR Blockers (5 µM), 5 µl of sample DNA at 5 ng/µl concentration and 14 µl of sterile water. For samples with DNA concentration below 5 ng/µl, 10 µl of sample DNA and 9 µl of sterile water were added to the reaction mixture. The reaction conditions for the PCR were: an initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 45 s, 75°C for 10 s, 50°C for 45 s, 72°C for 60 s, and a final extension at 72°C for 10 min. Following steps were conducted as described by Illumina [23].

The 16S libraries were sequenced on an Illumina MiSeq platform using a PE300 v3 kit at the Next generation sequencing platform of the Centre de recherche du CHU de Québec-Université Laval.

## 2.4. Bioinformatic analyses

Bioinformatic analyses were performed with QIIME2 [26] within Q2Pipe [27]. The detailed procedure is provided in Additional file 2. Briefly, sequences were denoised according to quality plots, primers were removed, and forward and reverse reads were merged using DADA2 [28]. Taxonomy was assigned using the SILVA database [29]. Amplicon sequence variants (ASVs) assigned to chloroplast and eukaryotes were filtered out, keeping only ASVs assigned to bacteria and archaea. Sequences were rarefied according to rarefaction curves at 3300 features for alpha and beta diversity analyses.

Subsequent analyses were performed in RStudio (version 4.4.2). A subset of methane-cycling communities was generated by selecting ASVs assigned to methanogenic and methanotrophic taxa with the function *subset\_taxa* (*Phyloseq* package). To do so, we searched the literature to generate a list of the main methanogenic-methanotrophic taxa (including facultative methanotrophs) to be included in the subset (Additional file 1: Table S1).

## 2.5. Statistical analyses

All statistical analyses were performed in RStudio (version 4.1.1).

First, we assessed the relative effects of tissue (leaf, wood and bark), compartment (leaf epiphytes, leaf endophytes, heartwood, sapwood and bark) and tree species on methane-cycling community composition, using NMDS (non-metric multidimensional scaling, *ordinate* function, *Phyloseq* package) and PERMANOVA (permutational multivariate analyzes of variance, *adonis2* function, *vegan* package) (objectives 1–2). NMDS was used to visualize dissimilarity between microbial communities according to different grouping factors (i.e. tissue, compartment and tree species). In PERMANOVA, we considered tissue, compartment and tree species as well as flood frequency (HFF and LFF) in the fixed factors, while block was considered as a random factor. The compartment was nested within tissue and the flood frequency nested within block to account for the structure of our experimental design. We considered the effect of the interactions between tree species, on which the experimental design was based on, and other fixed factors (tissue, compartment and flood frequency). Since the compartment is nested within the tissue, with unique compartment levels within each tissue, we tested the interactions between species and tissues, and between species and compartments in two separate models. We compared the

two alternative PERMANOVA models using the aikake criterion (*AICc\_permanova2* function, *AICcPermanova* package). We also tested differences in alpha diversity (Shannon index: *estimate\_richness* function, *Phyloseq* package) and total relative abundance of methanogens and methanotrophs among tissues and tree species (at the compartment level) by performing ANOVA followed by post-hoc analyses (*TukeyHSD* function, package *stats*). Then, pairwise PERMANOVA (*pairwise\_adonis* function, *vegan* package) were performed to test which species were different, in terms of methanogen-methanotroph composition, within each compartment. Differential abundance analyses (*ancombc2* function, *ANCOMBC* package) were then performed to assess which methanogenic and methanotrophic genera differed among tissues and tree species (at the compartment level). The ANCOM-BC was repeated by changing the reference group (species or tissue) to compare all levels with each other. Since ANCOM-BC is not designed to detect differences in taxa with structural zeros (i.e. taxa which is completely or nearly completely missing in some groups), differential abundance analyses were computed only for methanotrophic and methanogenic genera without structural zeros and did not allow to assess differences in genera which were absent in some groups. For objective 3, we tested the relationships between methanogenic-methanotrophic communities and traits of tissues and compartments by performing Mantel tests (*vegan* package). We then built mixed linear regression models and used procedure of stepwise regressions (function *stepAIC*, package *MASS*) to identify the best predictors of methanogens-methanotrophs relative abundances in the different phyllosphere compartments, among traits, species, block and flood frequency (LFF/HFF). We tested the correlations (*cor.test* function, package *stat*) between the relative abundance and the predictors in mixed linear regression models. Conditional relationships between the relative abundance of methanogens-methanotrophs and predictive variables were visualized using *visreg* (package *visreg*) and *ggplot2* functions.

## 3. Results

### 3.1. Methanogens and methanotrophs of tree phyllosphere tissues and compartments

Methanogens and methanotrophs were successfully detected in a high proportion of the phyllosphere samples investigated by 16S rRNA gene sequencing in this study (40.6% and 90.6% of samples for methanogens and methanotrophs respectively). Methanogens were mostly identified in sapwood and heartwood samples (Additional file 1: Fig. S2-S3), with *Methanobacterium* as the dominant genus across all samples. Other methanogens identified were assigned to genera *Methanobrevibacter*, *Methanomassilicoccus*, *Methanosaeta*, *Methanosarcina*, *Candidatus Methanogranum*, *Methanosphaerula* and the family *Methanomethylophilaceae*. Some tree individuals were characterized by the presence, and by higher proportions, of these less frequently observed methanogens in sapwood and heartwood (Additional file 1: Fig. S2-S3). Methanogens were also identified in some leaf epiphyte samples but at lower relative abundances (RA) (< 1%), with, again, *Methanobacterium* as the dominant genus identified followed by *Methanosarcina* (Additional file 1: Fig. S4). No sequences assigned to

methanogens were detected in leaf endophyte or bark samples. In contrast, methanotrophs were identified in all leaf epiphyte and endophyte samples (Additional file 1: Fig. S4-S5) as well as bark samples (Additional file 1: Fig. S6), but also in most sapwood and heartwood samples (Additional file 1: Fig. S2-S3). *Methylobacterium-Methylobacterium* was the dominant genus across all samples. Other methanotrophic genera identified were *Crenothrix*, *Methylobacter*, *Methylocapsa*, *Methylocella*, *Methylocystis*, *Methyloiligellaceae*, *Methylomicrobium*, *Methylomonas*, *Methylosorusula* and *pLW-20*. These other genera were identified in the methanotrophic communities of some tree individuals in variable proportions (Additional file 1: Fig. S2-S6).

Phyllosphere tissue (i.e. leaf, wood and bark) was the most discriminative factor of methanogenic-methanotrophic community composition among block, flood frequency, compartment and tree species (PERMANOVA  $p < 0.01$ , Table 1, Fig. 1). In addition to differing among phyllosphere tissues, methane-cycling community composition differed significantly according to species, flood frequency and block (PERMANOVA  $p < 0.01$ , Table 1). Effect of these factors was also observed for the whole prokaryotic community composition (Additional file 1: Fig. S7). The absence of significant effect of compartment in the PERMANOVA (Table 1) could be associated with the stronger effect of tissue masking the compartment effect. The interaction between compartment and species became significant when excluding the interaction with tissue (Table S2), although the PERMANOVA with the tissue-species interaction was better according to AIC values ( $AIC_{\text{tissue}} = -292.8$ ,  $AIC_{\text{compartment}} = -272.8$ ).

Considering the importance of tissue in structuring methanogenic-methanotrophic communities, we looked more closely at how these communities differed across leaf, wood and bark. Methanogens were found at a significantly higher relative abundance and alpha diversity (Shannon index) in wood when compared to leaf and bark (post-hoc  $p < 0.01$ , Additional file 1: Fig. S8-S9). Methanotrophs had a significantly higher relative abundance in leaves than in bark and wood (post-hoc  $p < 0.01$ , Additional file 1: Fig. S4), while the alpha diversity (Shannon index) was significantly higher in both leaf and bark than in wood (post-hoc  $p < 0.01$ , Additional file 1: Fig. S3). ANCOMBC indicated that wood differed from leaf and bark in terms of the relative abundance (RA) of the methanogen *Methanobacterium*, which was more abundant in wood, and the methanotroph *Methylobacterium*, which was more abundant in leaves and bark (ANCOMBC,  $p < 0.01$ ). Bark differed from leaves in terms of the RA of methanotrophs *Methylobacterium* and *Methylocella*, which were more abundant in leaves, and *Methylosorusula* which was more abundant in bark (ANCOMBC,  $p < 0.05$ ) (Additional file 1: Table S3).

Table 1  
Results from PERMANOVA testing the effect of flood frequency, tree species, tissue, compartment and block on methane-cycling microbial community composition.

| Factors                                                                                          | Df                                 | SumOfSqs | F     | Significance |
|--------------------------------------------------------------------------------------------------|------------------------------------|----------|-------|--------------|
| Flood frequency                                                                                  | 1                                  | 0.65     | 3.01  | **           |
| Species                                                                                          | 4                                  | 3.52     | 4.07  | ***          |
| Tissue                                                                                           | 2                                  | 29.69    | 68.58 | ***          |
| Compartment                                                                                      | 2                                  | 0.59     | 1.37  | ns           |
| Block                                                                                            | 5                                  | 1.90     | 1.76  | ***          |
| Flood frequency Block                                                                            | 5                                  | 1.67     | 1.55  | *            |
| Species:Flood frequency                                                                          | 4                                  | 0.97     | 1.12  | ns           |
| Species:Tissue                                                                                   | 8                                  | 4.54     | 2.62  | ***          |
| Overall                                                                                          | AIC: -292.8, R <sup>2</sup> = 0.51 |          |       |              |
| p-value < 0.001: ***; p-value < 0.01: **; p-value < 0.05: *; p-value < 0.1: .; p-value > 0.1: ns |                                    |          |       |              |

### 3.2. Inter-specific differences of phyllosphere methanogens and methanotrophs in various compartments of the phyllosphere

Considering that methanogens were absent from the leaf endophyte and bark compartments (Fig. 2), and that the interaction between tree species and phyllosphere compartment on methanogenic-methanotrophic community composition was significant when excluding the interaction between species and tissues (Additional file: Table S2), the effect of tree species on methanogenic-methanotrophic communities was investigated within each phyllosphere compartment.

Leaf epiphyte methanogenic-methanotrophic community composition of *Acer saccharinum* and *Ulmus americana* was different from that of *Fraxinus nigra* and *Populus* spp. (Pairwise PERMANOVA  $p < 0.05$ , although not significant after p-value adjustment, Additional file 1: Table S4; Fig. 2a-b). Alpha diversity (Shannon index) of leaf epiphytic methanotrophs of *Fraxinus nigra* was significantly higher than that of *Acer saccharinum* and *Populus* spp. (post-hoc  $p < 0.05$ , Additional file 1: Fig. S10). ANCOMBC showed that the relative abundance (RA) of the methanotrophic genus *Methylocella* in leaf epiphyte community of *Acer saccharinum* was significantly higher than that in *Populus* spp. and *Salix nigra* ( $p < 0.05$ , Additional file 1: Table S5-S6). No significant effect of tree species on the alpha diversity of leaf epiphytic methanogens, the RA of leaf epiphytic methanogenic genera, or the total relative abundance of methanotrophs and methanogens was detected (Additional file 1: Fig. S10-S11, Table S5-S6).

For the leaf endophytic compartment, where methanogens were not detected, the methanotrophic community composition of *Ulmus americana* was significantly different from that of *Fraxinus nigra* and *Populus* spp. (Pairwise PERMANOVA  $p < 0.05$ , Additional file 1: Table S4; Fig. 2c-d). The methanotrophic community composition of *Acer saccharinum* was also different from that of *Fraxinus nigra* (Pairwise PERMANOVA  $p < 0.05$ , although not significant after p-value adjustment). The alpha diversity and total relative abundance of leaf endophytic methanotrophs did not differ significantly between tree species (post-hoc  $p > 0.05$ , Additional file 1: Fig. S10-S11). However, ANCOMBC showed that the RA of *Methylobacterium* within the endophytic community of *Fraxinus nigra* was higher than that of *Acer saccharinum* ( $p < 0.05$ ).

The sapwood methane-cycling community composition, alpha diversity, total RA of methanogens and methanotrophs, and RA of methane-cycling genera did not differ significantly between tree species (Pairwise PERMANOVA  $p > 0.05$ , Additional file 1: Fig. S10-S11, Table S4-S6; Fig. 2e-f).

The heartwood methane-cycling community composition of *Populus* spp. differed from that of *Acer saccharinum* and *Fraxinus nigra* (Pairwise PERMANOVA  $p < 0.05$ , although not significant after p-value adjustment, Additional file 1: Table S4; Fig. 2g-h). The heartwood community composition of *Salix nigra* was also distinct from that of *Fraxinus nigra* (Pairwise PERMANOVA  $p < 0.05$ , although not significant after p-value adjustment). In addition, the alpha diversity and total relative abundance of heartwood methanotrophs were higher in *Fraxinus nigra* than in *Ulmus americana* and *Salix nigra* (for alpha diversity only) (post-hoc  $p < 0.05$ , Additional file 1: Fig. S10-S11). No difference in the RA of heartwood methanotrophic genera between tree species was detected (ANCOMBC  $p > 0.05$ , Additional file 1: Table S5-S6). In contrast, while the alpha diversity of heartwood methanogens did not differ significantly between species (post-hoc  $p > 0.05$ ), ANCOMBC showed that *Populus* spp. differed from *Fraxinus nigra* and *Acer saccharinum* by its higher RA of *Methanomassilicoccus* ( $p < 0.05$ , although not significant after p-value adjustment, Additional file 1: Table S5-S6) in addition to having a higher total relative abundance of heartwood methanogens than *Fraxinus nigra* (post-hoc  $p < 0.05$ , Additional file 1: Fig. S11). *Populus* spp. was also characterized by the presence of *Candidatus Methanogranum* in the heartwood or sapwood of some individuals (Additional file 1: Fig. S2-S3).

For bark, where methanogens went undetected, the methanotrophic community composition of *Acer saccharinum* was significantly different from that of *Fraxinus nigra*, *Ulmus americana* and *Populus* spp., while *Salix nigra* differed significantly from *Fraxinus nigra* and *Populus* spp., and *Populus* spp. differed significantly from *Ulmus americana* (Pairwise PERMANOVA  $p < 0.05$ , Additional file 1: Table S4; Fig. 2i-j). In addition, *Populus* spp. exhibited a higher alpha diversity of bark methanotrophs than *Acer saccharinum* and *Salix nigra*, and a higher total relative abundance of bark methanotrophs than *Salix nigra* and *Ulmus americana* (post-hoc  $p < 0.05$ , Additional file 1: Fig. S10-S11). The bark methanotrophic community of *Acer saccharinum* differed notably by the higher relative abundance of *Methylocella* compared to *Populus* spp. and *Salix nigra* (ANCOMBC  $p < 0.05$ , Additional file 1: Table S5-S6). It was also characterized by the presence of *Methylobacter*, *Methylocapsa* and *Methylobacterium* identified in some individuals (Fig. 2j; Additional file 1: Fig. S6).

### 3.3. Tree traits and their relationships with phyllosphere methanogens-methanotrophs

We then looked at how species traits varied among tree species and influenced the methane-cycling communities. Relationships between traits and methanogen-methanotroph community composition of phyllosphere compartments were first assessed by performing Mantel tests. We then examined the relationships between traits and the relative abundance of methanogens and methanotrophs across the different phyllosphere compartments using linear regression models.

#### 3.3.1. Inter-specific variations in tree traits

Leaf pH and humidity differed significantly among tree species (ANOVA  $p < 0.05$ ) (Additional file 1: Table S7). Specifically, *Acer saccharinum* had a lower leaf pH than other species (post-hoc  $p < 0.05$ ). Among the other species, *Salix nigra* had a lower leaf pH than *Ulmus americana* and *Fraxinus nigra* (post-hoc  $p < 0.05$ ). *Fraxinus nigra* had a higher leaf humidity than *Acer saccharinum* (post-hoc  $p < 0.05$ ). The LMA of *Populus* spp. and *Fraxinus nigra* was slightly higher than that of *Acer saccharinum*, *Ulmus americana* and *Salix nigra* although the difference was not significant (ANOVA  $p = 0.09$ ).

Wood humidity, pH and density differed significantly among tree species (ANOVA  $p < 0.01$ ) (Additional file 1: Table S7). Specifically, *Populus* spp. and *Salix nigra* had a higher heartwood humidity than *Fraxinus nigra* and *Acer saccharinum* (post-hoc  $p < 0.05$ ). *Populus* spp. also had a higher sapwood humidity than *Fraxinus nigra*. *Fraxinus nigra* had a lower heartwood pH than other tree species, while *Populus* spp. had a higher heartwood pH than *Acer saccharinum*, *Fraxinus nigra* and *Salix nigra* (post-hoc  $p < 0.05$ ). In addition, *Fraxinus nigra* had a lower sapwood pH than *Salix nigra* and *Ulmus americana* (post-hoc  $p < 0.05$ ). Heartwood density of *Ulmus americana* was higher than that of *Populus* spp., *Salix nigra* and *Acer saccharinum*, while sapwood density of *Ulmus americana* and *Fraxinus nigra* was higher than that of *Populus* spp. and *Salix nigra* (post-hoc  $p < 0.05$ ).

Bark pH and density differed significantly between tree species (ANOVA  $p < 0.01$ ) (Additional file 1: Table S7). The bark pH of *Acer saccharinum* was lower than that of *Fraxinus nigra*, *Salix nigra* and *Ulmus americana*, and bark pH of *Populus* spp. was significantly lower than that of *Salix nigra* and *Ulmus americana* (post-hoc  $p < 0.05$ ). In addition, the bark density of *Acer saccharinum* was higher than that of *Fraxinus nigra*, *Salix nigra* and *Ulmus americana* (post-hoc  $p < 0.05$ ). Bark humidity did not differ significantly between tree species (ANOVA  $p > 0.05$ ).

#### 3.3.2. Relationships between traits and the methane-cycling community

We identified relationships between tree traits and the phyllosphere methane-cycling community composition. First, the composition of the leaf epiphytic methanogen-methanotroph community was significantly correlated with leaf pH (Mantel  $R^2 = 0.13$ ,  $p < 0.05$ ), while the composition of the leaf

endophytic methanotroph community was significantly correlated with leaf humidity (Mantel  $R^2 = 0.18$ ,  $p < 0.05$ ) (Table 2). The composition of the sapwood and heartwood methanogen-methanotroph community was correlated with pH, although this correlation was only marginally significant (Mantel  $R^2 = 0.17$  for sapwood and 0.10 for heartwood,  $p = 0.07$ ) (Table 2). The composition of the bark methanotroph community was significantly correlated with bark density and pH (Mantel  $R^2 = 0.21$  and 0.26,  $p < 0.01$ ) (Table 2).

Table 2

Mantel tests results for the correlation between methanogenic-methanotrophic communities of tree phyllosphere compartments (leaf epiphytes, leaf endophytes, sapwood, heartwood and bark) and tree traits.

| Factors                                                                                         | Mantel R | P-value   | Significance   | Mantel R | P-value | Significance |
|-------------------------------------------------------------------------------------------------|----------|-----------|----------------|----------|---------|--------------|
| Leaf epiphyte                                                                                   |          |           | Leaf endophyte |          |         |              |
| All                                                                                             | 0.142    | 0.028     | *              | 0.142    | 0.053   | .            |
| LMA                                                                                             | 0.104    | 0.097     | .              | 0.055    | 0.251   | ns           |
| pH                                                                                              | 0.134    | 0.033     | *              | 0.005    | 0.453   | ns           |
| Humidity                                                                                        | 0.041    | 0.274     | ns             | 0.180    | 0.025   | *            |
| Sapwood                                                                                         |          |           | Heartwood      |          |         |              |
| All                                                                                             | 0.231    | 0.030     | *              | -0.005   | 0.504   | ns           |
| pH                                                                                              | 0.172    | 0.067     | .              | 0.101    | 0.065   | .            |
| Density                                                                                         | 0.023    | 0.371     | ns             | -0.085   | 0.907   | ns           |
| Humidity                                                                                        | 0.104    | 0.126     | ns             | 0.022    | 0.352   | ns           |
| DBH                                                                                             | 0.093    | 0.176     | ns             | -0.037   | 0.637   | ns           |
| Bark                                                                                            |          |           |                |          |         |              |
| All                                                                                             | 0.328    | 1.000E-04 | ***            |          |         |              |
| pH                                                                                              | 0.259    | 1.000E-04 | ***            |          |         |              |
| Density                                                                                         | 0.206    | 0.002     | **             |          |         |              |
| Humidity                                                                                        | 0.082    | 0.133     | ns             |          |         |              |
| p-value < 0.001: ***; p-value < 0.01: **; p-value < 0.05: *; p-value < 0.1:.; p-value > 0.1: ns |          |           |                |          |         |              |

We also identified relationships between tree traits and the relative abundance of methanogens and methanotrophs of the phyllosphere using mixed linear regression models (Additional file 1: Table S8-S10).

The mixed linear regression model which best predicted the relative abundance (RA) of leaf epiphytic methanogens included block, species, LMA and DBH as predictors ( $R^2 = 0.27$ ,  $p = 0.02$ ). Epiphytic methanogen RA was correlated positively with LMA and negatively with DBH (Fig. 3a). In this model, *Fraxinus nigra* had a significant negative effect on methanogen RA (Additional file 1: Table S9, Fig. S12), although the RA of leaf epiphytic methanogens of *Fraxinus nigra* was not significantly different from that of other species according to the post-hoc analysis ( $p > 0.05$ ). The RA of leaf epiphytic methanotrophs was best predicted by leaf pH, to which it was positively correlated ( $R^2 = 0.06$ ,  $p = 0.04$ ) (Fig. 3a).

Methanotroph RA in leaf endophytes was best predicted by the tree species, block, LMA, DBH and leaf humidity ( $R^2 = 0.41$ ,  $p = 0.02$ ). In this model, methanotroph RA was negatively correlated with leaf humidity and DBH (Fig. 3a). *Ulmus americana* had a significantly lower RA of leaf endophytic methanotrophs than *Populus* spp. and *Fraxinus nigra* (post-hoc  $p < 0.05$ , Additional file 1: Fig. S12).

The RA of sapwood methanogens was best predicted by species, DBH and sapwood humidity, although the model was not significant ( $R^2 = 0.22$ ,  $p = 0.10$ ) (Fig. 3b, Additional file 1: Fig. S13). In this model, *Ulmus americana* had a significant negative effect on methanogen RA (Additional file 1: Table S9, Fig. S13), although the RA of sapwood methanogens of *Ulmus americana* was not significantly different from that of other species according to the post-hoc analysis ( $p > 0.05$ ). The model of sapwood methanotroph RA included sapwood humidity but was also not significant ( $R^2 = 0.03$ ,  $p = 0.13$ ) (Fig. 3b). Methanotroph RA was negatively correlated with sapwood humidity (Fig. 3b).

In heartwood, methanogen RA was best predicted by heartwood pH, humidity, DBH and flood frequency ( $R^2 = 0.45$ ,  $p < 0.01$ ). Methanotroph RA was best predicted by block, species, heartwood humidity, pH and DBH ( $R^2 = 0.45$ ,  $p < 0.01$ ). In this model, *Populus* spp. and *Salix nigra* had a significant positive effect on methanotroph RA (Additional file 1: Table S9, Fig. S13), although the RA of heartwood methanotrophs of *Populus* spp. and *Salix nigra* was not significantly different from that of other species according to the post-hoc analysis ( $p > 0.05$ ). In these models, methanogen RA was positively correlated with pH and DBH, while methanotroph RA was negatively correlated with heartwood humidity (Fig. 3b).

Bark methanotroph RA was best predicted by species and bark pH, to which it was negatively correlated ( $R^2 = 0.41$ ,  $p < 0.01$ ) (Fig. 3b). *Acer saccharinum* had a significantly lower RA of bark methanotrophs than *Populus* spp. (post-hoc  $p < 0.01$ , Additional file 1: Fig. S14).

## 4. Discussion

### 4.1. Phyllosphere tissue structures the methane-cycling community

Although the contrasting methane-cycling communities of various tree tissues and their potential role in regulating methane fluxes have been previously described in the literature, our study provides, for the first time, data on methanogens and methanotrophs across multiple compartments and tissues

(including bark) for several broadleaf tree species commonly found in floodplains, thereby expanding the current knowledge on the diversity and distribution of this important group of microorganisms in the tree phyllosphere. Our results showed that the tree phyllosphere methane-cycling community is, in the first place, structured by tissue type, indicating that the role of the phyllosphere microbiota in the regulation of tree CH<sub>4</sub> fluxes potentially differs between leaf, wood and bark.

The dominance of methanogens over methanotrophs in wood, which could be associated with anoxic conditions, suggests a potential role of wood microbiota in intrinsic methane production, as previously demonstrated for *Populus* spp. [6, 8, 10]. The RA of methanogens was much higher in this tissue type, reaching > 30% of the bacterial and archaeal sequences in some samples, while the RA of methanotrophs was generally < 1% (Additional file 1: Figure S6-S7). These values were comparable to those reported by Yip et al. [6], with RA of the methanogen *Methanobacterium* reaching over 40% in some samples and the RA of methanotrophs ranging from 0.04% in heartwood to 0.1% in sapwood. The wood methanogenic communities of the five tree species studied here were dominated by the same genus, *Methanobacterium*, as reported previously for *Populus* spp. [6, 8, 11]. As discussed in these studies, the dominance of *Methanobacterium* could be associated with its tolerance to O<sub>2</sub>, which can be available in wood, although its concentration typically decreases from bark to heartwood. Some of the other genera detected in wood, such as *Methanobrevibacter*, *Methanomassiliicoccus* and *Methanosarcina*, were previously observed in this tissue type [8, 30]. Additional methanogens were identified in our study (*Methanosaeta*, *Candidatus Methanogranum* and *Methanosphaerula*), expanding the diversity of taxa reported in wood.

Inversely, the dominance of methanotrophs over methanogens in leaf and bark, which could be explained by a higher oxygen availability, suggests that leaf and bark microbiota could play a role in reducing tree methane emissions. Methanotrophic taxa identified in leaf tissues (epiphytes and endophytes) in our study (*Methylobacterium*, *Methylocella*, *Methylocystis* and *Methylocapsa*) are commonly found in leaves of diverse plant species [31, 32, 33]. Methylophiles are well-adapted to the leaf environment, notably due to their capacity to grow on methanol (or methane) released by plants and form mutualistic associations with plants, whose stress tolerance and growth are enhanced by metabolites released by these bacteria [31, 34]. *Methylobacterium* is a major bacterial inhabitant of the phyllosphere due to its ability to use plant-produced methanol as a substrate [31, 35]. This can thus explain the dominance of this genus in the methanotrophic communities across samples from various tree species and phyllosphere tissues, including bark, in which it had not been reported previously. Bark was also characterized by higher RA of the facultative methanotroph *Methylosarcina*, previously found in the core microbiome of a lichen species in boreal regions [36] and in acidic tundra wetland soils [37], but not in the tree phyllosphere. Interestingly, the methanotrophs detected at higher RA in bark samples in our study were distinct from those identified in the few previous studies (e.g. *Methylobacterium* and *Methylosinus*) analyzing this type of tissue [12, 31], likely because of differences in tree species and environmental conditions. However, the dominance of acidophilic methanotrophs in bark appeared as a common characteristic of this tissue type that should be further explored in future studies.

Like *Methylosula*, several of the methanotrophic taxa detected in our study, such as *Methylocapsa* and *Methylocella*, are facultative methanotrophs that can use other substrates than methane. Assessing their methane oxidation activity, for instance through gene expression analysis, will therefore be necessary to confirm their role in methane oxidation within the phyllosphere. Due to the exposure of leaf and bark to ambient air, and thus to atmospheric methane concentrations, methanotrophic communities in these tissues may also be composed of high affinity methanotrophs that would not have been identified through their 16S rRNA gene [14, 38]. For instance, using *pmoA* sequencing, Jeffrey *et al.* [12] identified an uncultivated high-affinity methanotroph cluster in bark samples. However, considering that this cluster had a lower RA than low-affinity methanotrophs and that high-affinity methanotrophs were not detected by Iguchi *et al.* [31] in plant tissues of different species using *pmoA* sequencing, high-affinity methanotrophs are likely not a dominant group in the tree phyllosphere.

Surprisingly, methanogens were identified in the leaf epiphytic compartment of trees in our study, although their relative abundance was much lower than in wood (< 0,05%). Some of the methanogens identified as leaf epiphytes (*Methanobacterium* and *Methanosarcina*) are known for their resistance to oxidation, which could explain their presence in this oxygenic habitat [6, 39]. Methanogens were also identified in *Picea abies* needles by Putkinen *et al.* [14] although they were from other genera (i.e. *Methanoregula*, *Methanotrix*). These differences could be explained by the distinct habitat of leaves and needles.

Interestingly, the overall 16S microbial community also differed among phyllosphere tissues. This suggests that the methane-cycling community is modulated by tissue conditions which also structure the whole microbial community. Cregger *et al.* [40] previously demonstrated that the tissue/habitat class (i.e. leaf, stem, roots and soil) was the main driver of tree microbial communities, as observed in our study, indicating tissue-specific filters. Indeed, there are different niches structuring microbial communities in trees, notably in terms of nutrient and oxygen availability, light exposure, biochemical compounds and carbon sources [33, 40–42]. For instance, anoxic conditions which can form in heartwood can select microbes with anoxic metabolism such as fermentation, nitrogen fixation, and methanogenesis [40]. Otherwise, the methanol, metabolically produced by the plant and emitted at the leaf surface, can favor the methylophilic lifestyle [42].

## 4.2. Species and their traits modulate the tree phyllosphere methane-cycling communities

In addition to the effect of tissue type, our results demonstrated that the methane-cycling communities, as well as the overall 16S communities, differed according to tree species. This could imply that the role of the phyllosphere microbiota in tree methane flux regulation differs among species. We identified several key traits modulating the composition of methane-cycling communities. Tree species and traits also played a role in modulating the relative abundance of methane-cycling microorganisms in leaves, wood and bark, as demonstrated by our regression models. The importance of tree species and their traits in structuring the microbial communities of tree phyllosphere demonstrated in the studies of

Laforest-Lapointe et al. [16] and Lajoie et al. [43] is expanded here to methane-cycling communities. This informs on how tree composition in floodplain and other wetlands could have an impact on methane emissions.

### 4.2.1. Tissue pH

Tissue pH was identified as a key trait explaining the composition of methane-cycling communities of leaves, wood and bark. Tissue pH can influence the methane-cycling community by selecting methanogenic and methanotrophic taxa according to their pH optimum and tolerance [19]. Zhao et al. [44] have previously shown that pH is determinant in the differentiation of methanotroph niches in soils and that it modulates the composition of the active methanotrophic communities. Our results suggest a similar role of pH in the tree phyllosphere. For instance, the lower leaf and bark pH of *Acer saccharinum* may explain its distinct methanotrophic community, notably characterized by a higher relative abundance of the acidophilic facultative methanotroph *Methylocella* (pH optimum: 5–6) [45]. Although very few studies have investigated the methane-cycling communities of bark, Jeffrey et al. [12] have demonstrated the important role played by the acidophilic methanotrophic community of the *Melaleuca quinquinervia* bark in reducing tree methane emissions, indicating that pH might be a key factor regulating methane oxidation in this tissue type. In addition, the lower heartwood pH of *Fraxinus nigra* (4.8) may explain its distinct methane-cycling community, notably characterized by a higher alpha diversity of methanotrophs, compared to *Populus* spp. which had a higher heartwood pH (7.7) and a higher RA of the methanogen *Methanomassiliococcus*. Of note, the genus *Methanomassiliococcus* is a methanol-reducing hydrogenotrophic methanogen which could also be influenced by the availability of H<sub>2</sub>, methanol and other methylated substrates in wood [8, 47]. We suggest that the concentrations of these compounds in wood might also modulate the methane-cycling communities of tree species and should be investigated in future studies.

Tissue pH was also a predictor of the relative abundance of methanogens-methanotrophs within the tree phyllosphere. Interestingly, while leaf epiphytic methanotroph RA was positively correlated to leaf pH, a negative correlation was observed in bark and heartwood (although not statistically significant for heartwood). These differences could be linked to the distinct ranges of pH observed in each tissue type. In leaves (pH 4.3–5.8), methanotroph tolerance and optimum would be more compatible with upper range values, while in bark (pH 5.5–6.5) and heartwood (pH 4.8–7.7), lower range values might be more favorable to methanotrophs. Indeed, the optimal pH for methanotrophy has been shown to be 5.0–6.5 [20]. This negative correlation between pH and the RA of methanotrophs in wood and bark, and the positive correlation of pH with the RA of wood methanogens, suggest that lower wood and bark pH might be more favorable to methanotrophy and less favorable to methanogenesis. This could be due to the higher tolerance of methanotrophs than methanogens to lower pH [19]. Tissue pH could thus be a key tree trait associated with the regulation of intrinsic methane production and consumption.

### 4.2.2. Tissue humidity

Humidity was identified as a trait explaining the composition of leaf endophytic methanotrophs. The effect of humidity may be associated with the modulation of oxygen availability in tissues. Variability in this trait could explain some differences observed in methane-cycling communities among species. For instance, the higher leaf humidity of *Fraxinus nigra* may explain its distinct leaf endophytic methanotrophic community.

Humidity was also a key predictor of the relative abundance of wood and leaf endophytic methanotrophs. Methanotroph RA was negatively correlated with humidity, which is again probably linked to the effect of humidity on O<sub>2</sub> availability. The higher wood humidity of *Populus* spp., along with its higher pH, may explain its higher total RA of heartwood methanogens compared to other species. Higher wood humidity can generate anoxic conditions favorable to methanogens and to methane production, as observed in the study of Wang et al. [46]. Inversely, the lower wood humidity and pH of *Fraxinus nigra* may explain its higher total RA of heartwood methanotrophs.

### 4.2.3. Tree diameter

While not identified as a strong driver of the methane-cycling community composition, tree diameter (DBH) was found to be significantly correlated to the RA of leaf and wood methanogens-methanotrophs. The RA of leaf endophytic methanotrophs and of leaf epiphytic methanogens were inversely correlated with DBH. This relationship might be associated with the influence of tree size/age on leaf colonization. Younger/smaller trees may be prone to higher leaf colonization by soil methanotrophs and methanogens due to greater proximity between foliage and soil as suggested by Gorgolewski et al. [4].

Inversely, the positive correlation between methanogen RA in heartwood and DBH indicates favorable conditions to the methanogens in bigger/older trees which can be associated with lower diffusion of O<sub>2</sub> inside the trunk. This may result in higher methane production and stem emissions in older individuals, which agrees the finding of Wang et al. [46] who observed a positive relationship between heartwood size and CH<sub>4</sub> concentrations in stems. Yip et al. [6] also identified tree diameter as a predictor of wood methanogen RA. However, in their study, methanogen RA was negatively correlated with DBH and neither wood pH nor humidity were important predictors of methanogen RA, which contrasts from our findings. The importance of these traits in predicting wood methanogen RA in our study may come from the variability of traits between the different species. In contrast, the study from Yip et al. [6] was conducted exclusively on the species *Populus deltoides*, indicating that trends might differ at the intra-specific level compared to the inter-specific level.

### 4.2.4. Other traits

For leaves, LMA was also a trait explaining the relative abundance of epiphytic methanogens, which were positively correlated with LMA. LMA is linked to the resource uptake strategy of the species and is known to influence the nutrient availability both inside and on the surface of leaves, which in turn influences the leaf microbial communities [16, 43]. It is also a proxy for other traits such as carbohydrate content which can influence the microbial communities [48]. No significant difference in LMA among

species was observed in our study, which limits the potential to link the trait's effect on the methane-cycling community to the species resource acquisition strategies. Analyzing the relationship between this trait and methane-cycling community in species with a wider range of LMA, as well as measuring other LMA-associated traits, could help better assess its effect on leaf methanogens and methanotrophs.

Bark density was also a tree trait modulating the composition of bark methanotrophs. Thus, the higher bark density of *Acer saccharinum* could have contributed, along with pH, to its distinct bark methanotrophic community in comparison to the other tree species.

## **4.2.5. Other predictors of phyllosphere methanogens/methanotrophs relative abundance**

Tree species also explains a part of the variance along with tree traits in the models of methanogen-methanotroph relative abundance, which indicates that the species can have an effect beyond the traits discussed here. This could mean that other species-traits, such as metabolite composition of tissues, can structure methane-cycling communities.

Apart from species and traits, the block was also a predictive variable of the relative abundance of leaf and heartwood methane-cycling microorganisms in our regression models. Laforest-Lapointe et al. [16] demonstrated the influence of site, along with species, as a main driver structuring the leaf microbiome of trees. This can indicate the contribution of phyllosphere colonization by local microorganisms to the tree methanogenic-methanotrophic communities. Moreover, the sites are associated to conditions that determine soil methane production and microbial communities which can in turn influence the tree microbiota through microbes' transportation via transpiration as well as through the setting of conditions for microbial mechanisms in trees (e.g., via the transport of methane inside trees and modulation of tree traits). This could also explain the effect of flood frequency on the relative abundance of heartwood methanogens. Microbial dispersal between neighboring trees may also have played a role in homogenizing tree methanogen-methanotroph communities at each block, which are also structured by the dominant tree species of the stand [49].

## **5. Conclusion**

Our study shows the prevalence of methanogenic-methanotrophic in the tree phyllosphere and highlights the importance of tissues and their characteristics in structuring these communities, acting in concert with species and traits. Different traits (e.g., leaf, sapwood, and heartwood pH and humidity, as well as bark pH and density) were found to modulate the methanogenic and methanotrophic communities of the tree phyllosphere. Tree species differing in these key traits could therefore be associated with distinct methanogenic-methanotrophic communities, and in turn with differential microbial methane production/consumption potentials. Studies combining flux measurements with microbial community analyses are needed to assess whether these differences in tree methanogenic-methanotrophic communities are responsible for differential CH<sub>4</sub> fluxes among tree species. Analysis of

methanogenesis/methanotrophy gene expression could also help better assess the contribution of the tree microbial communities to methane fluxes. In continuation with our study, future work should include several tree species with a higher trait variability to better understand the structure of the tree methane-cycling microbiota. We suggest that secondary metabolites of tissues and their relationships with methane-cycling communities would be of particular interest to investigate in future studies.

Overall, we advocate that a better characterization of the tree phyllosphere methane-cycling microbial communities, along with methane-flux measurements, is necessary to understand the role of the tree microbiota in CH<sub>4</sub> cycling and its potential contribution to climate mitigation strategies. Selecting tree species with a strong potential for methanotrophy based on their traits for reforestation could be an interesting solution to mitigate methane emissions and help achieve the IPCC targets of 30% reduction in methane emissions by 2035.

## Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Sequence data were deposited in the Sequence Read Archive within BioProject PRJNA1196399. All other data generated or analyzed during this study are included in this article and its additional files. Codes used for sequence data processing and statistical analyses are available in Additional file 2.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by the Genomics Research and Development Initiative (GRDI), the Fonds de recherche du Québec-Nature et technologies (FRQNT), the Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG), the Ministère de l'Environnement, de la Lutte contre les changements climatiques, de la Faune et des Parcs (MELCCFP), the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation (MAPAQ) and the Pôle d'expertise multidisciplinaire en gestion durable du littoral du lac Saint-Pierre.

Authors' contributions

MAM, CM and VM designed the study. MAM collected and processed the samples for microbial and tree trait analyses. MJM and MAM prepared the libraries for amplicon sequencing. MAM did bioinformatics and statistical analyses. MAM, CM and VM wrote the main manuscript text. All authors read and approved of the final manuscript.

## Acknowledgements

The study took place on territories of Abenakis and Attikamekw to whom we are thankful. We would also like to thank Saylena Fay, Mathieu Michaud and Marilie Trudel for their participation in the field sampling campaign and the team of Christine Martineau's laboratory for their support in the analyses.

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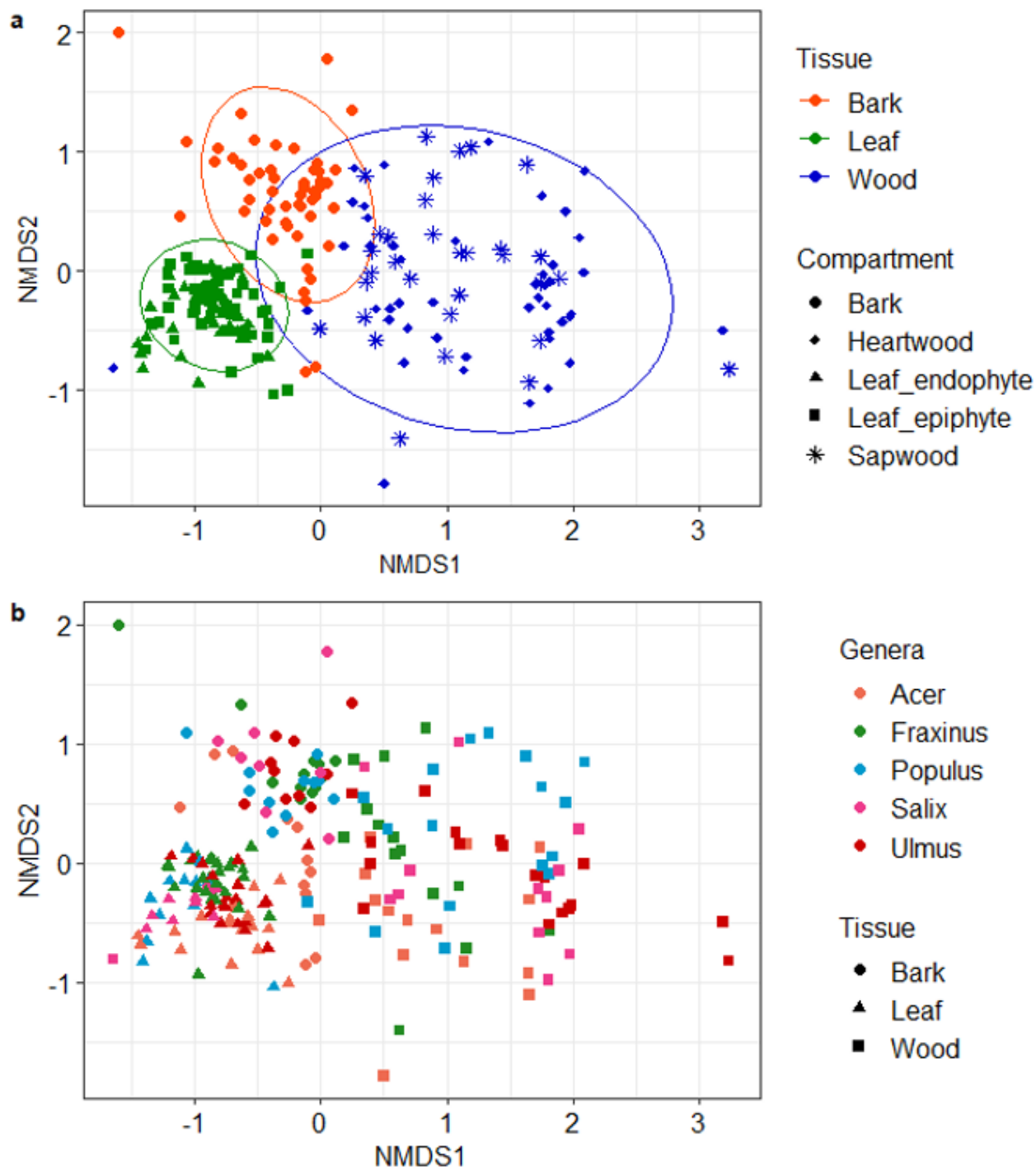
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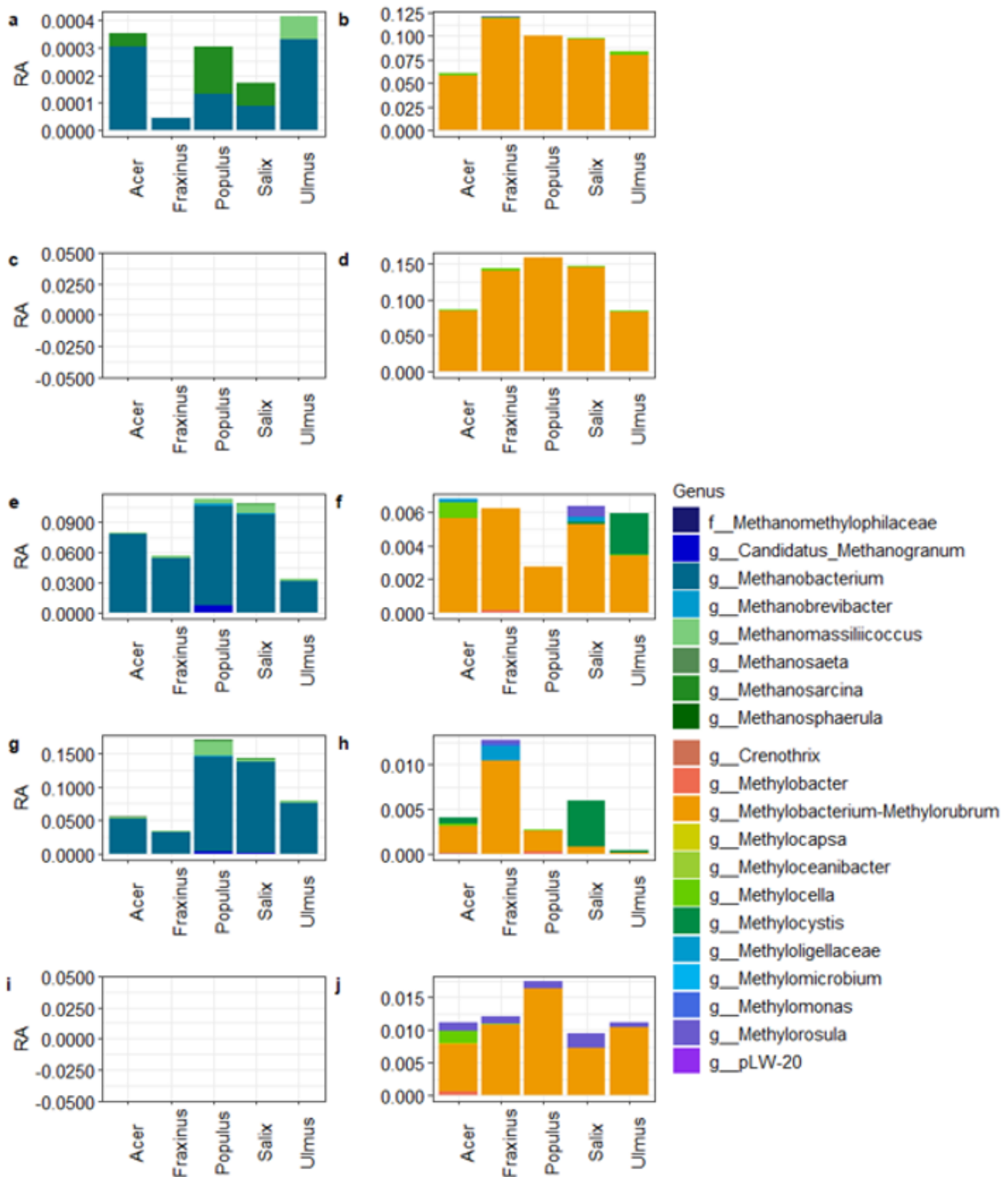
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## Figures



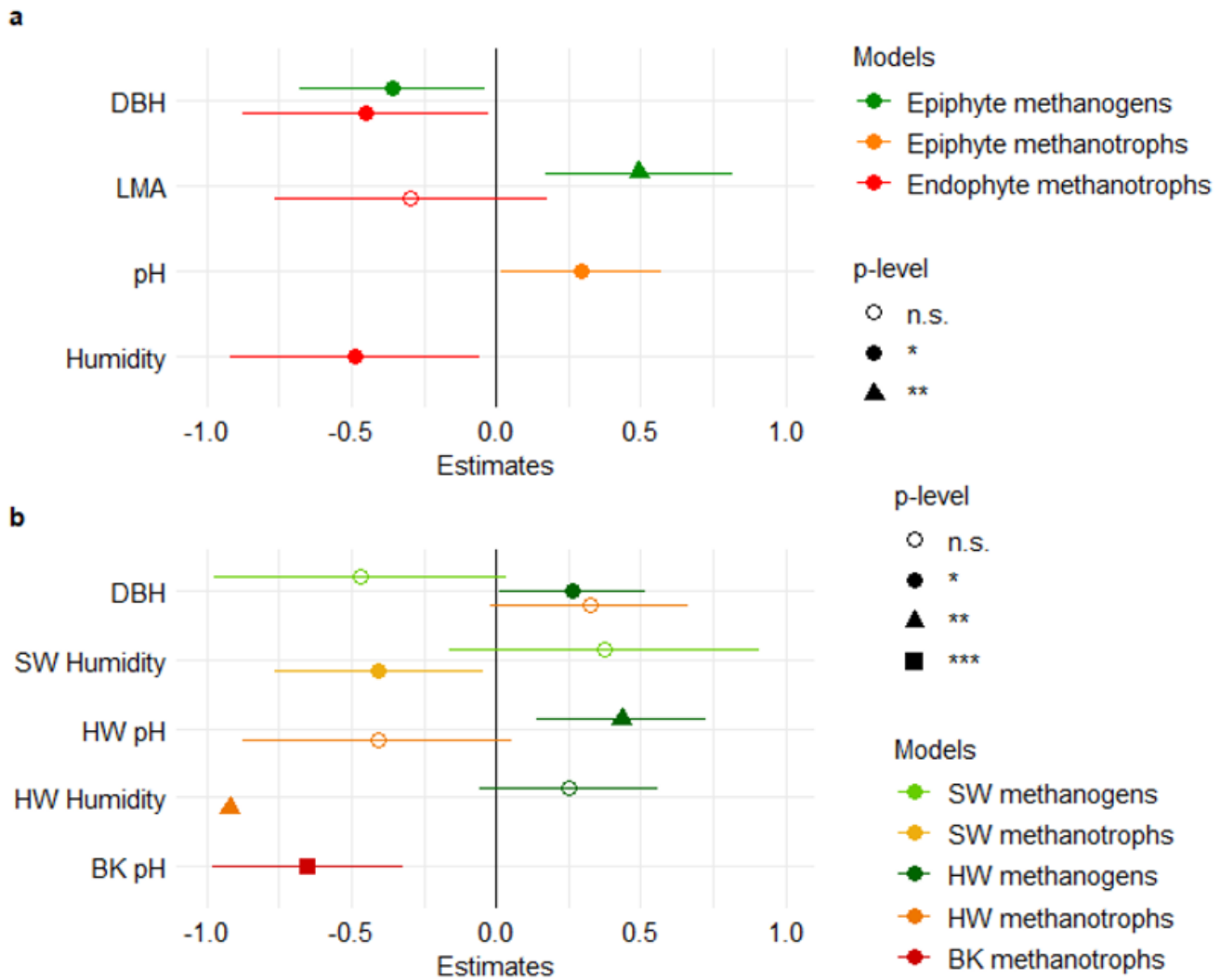
**Figure 1**

NMDS ordination (Bray-Curtis dissimilarity) of tree methanogen-methanotroph communities among phyllosphere tissues (bark, leaf and wood) (a) and tree species (b).



**Figure 2**

Methanogenic (a, c, e, g, i) and methanotrophic (b, d, f, h, j) communities of leaf epiphytes (a, b), leaf endophytes (c, d), sapwood (e, f), heartwood (g, h) and bark (i, j) of tree species (*Acer saccharinum*, *Fraxinus nigra*, *Populus* spp., *Salix nigra* and *Ulmus americana*) in terms of the relative abundance (RA) of methanogenic and methanotrophic genera in the 16S microbial community.



**Figure 3**

Conditional relationships ( $R^2$  estimates) between traits and the relative abundance of leaf epiphytes, leaf endophytes, sapwood (SW), heartwood (HW) and bark (BK) methanogens-methanotrophs in the regression models.

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