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Short Communication

Methanotrophs are core members of the diazotroph community in decaying Norway spruce logs

Raisa Mäkipää[a,*], Sanna M. Leppänen[a], Sonia Sanz Munoz[a], Aino Smolander[a], Marja Tiirola[b], Tero Tuomivirta[a], Hannu Fritze[a]

[a] Natural Resources Institute Finland, Finland
[b] University of Jyväskylä, Finland

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ABSTRACT

Dead wood is initially a nitrogen (N) poor substrate, where the N content increases with decay, partly due to biological N_2 fixation, but the drivers of the N accumulation are poorly known. We quantified the rate of N_2 fixation in decaying Norway spruce logs of different decay stages and studied the potential regulators of the N_2-fixation activity. The average rate for acetylene reduction in the decaying wood was 7.5 nmol ethylene $g^{-1}d^{-1}$, which corresponds to 52.9 μg N $kg^{-1}d^{-1}$. The number of *nifH* copies (g^{-1} dry matter) was higher at the later decay stages, but no correlation between the copy number and the *in vitro* N_2 fixation rate was found. All recovered *nifH* sequences were assigned to the order Rhizobiales, and therein mostly (60%) to methane oxidizing genera. We confirm that nitrogen fixing methanotrophs are present in all the wood decay phases and suggest that their interaction between methane producing organisms in decaying wood should be further studied.

In boreal forests with nitrogen (N) supply deficiency, asymbiotic N_2 fixation occurs in the dead wood (Brunner and Kimmins, 2003; Hicks et al., 2003; Rinne et al., 2017), the bryophyte (DeLuca et al., 2002; Leppänen et al., 2013) and the litter and soil layers (Todd et al., 1978; Vitousek and Hobbie, 2000). During the dead wood decay, where fungal communities proliferate, both the N concentration and the total amount of N in the wood increase along with the decay phase (Rajala et al., 2012) and the biological N_2 fixation, which explains part of this increase (Rinne et al., 2017). In decaying wood, the N cycling processes and fungi-driven decomposition are tightly linked (Bebber et al., 2011), and the transfer of fixed N_2 to the fungal biomass has been proven (Weisshaupt et al., 2011). However, the relationship between the diazotroph community and the rate of N_2 fixation in dead wood is poorly known. We hypothesize that the N_2 fixation rate and the abundance of diazotrophic bacteria increase with the mass loss of decaying wood.

Our study site was an unmanaged Norway spruce dominated forest in Sipoo, Finland (60°28′N, 25°12′E). A full description of the study site, sampling, decay classification, acetylene reduction assay (ARA), and molecular analyses are given in Rajala et al. (2012) and in the Supplementary material. Briefly, wood discs were sampled from fallen dead trees representing different decay stages from recently fallen trees (decay stage 1) to late decay stage (5) when the logs were very soft and covered by bryophytes. The assessment of the N_2 fixation by acetylene reduction assay (ARA) in 32 dead fallen Norway spruce (*Picea abies* L. (Karst)) logs (using four replicates per sample log) followed the methods described in Rinne et al. (2017). The methods of DNA extraction and purification, denaturing gradient gel electrophoresis (DGGE; gradient range 35–75%; 75 V; 60°C; 16 h), DGGE band excision and Sanger sequencing followed Rajala et al. (2012), except for that the DNA was extracted from 100 mg of lyophilized and milled material with the Nucleospin Soil extraction kit (Macherey-Nagel, Germany). The SL1 lysis buffer was used, but no SX enhancer. For DGGE, the *nifH* was first amplified for 40 cycles by qPCR was followed by rounds of end-point PCR (10–26 cycles depending on the previous C_t values) using the primers PolF with a GC-clamp and PolR (Poly et al., 2001). The primers and qPCR conditions used in quantifying the bacterial and archaeal 16S rRNA and bacterial *nifH* genes are listed in Supplementary Table 1.

The average rate of the acetylene reduction was 7.55 nmol ethylene g^{-1} d^{-1}, which gives 52.9 μg N kg^{-1} d^{-1}. The number of the *nifH* copies (g^{-1} dry matter) and detected DGGE bands correlated negatively with the wood density ($r = -0.43$, $p = 0.014$; and $r = -0.52$, $p = 0.002$, respectively) and positively with the wood moisture ($r = 0.54$, $p = 0.002$ and $r = 0.44$, $p = 0.011$). The measured rate of N_2 fixation was higher at the intermediate stage of decay (Fig. 1). The number of *nifH* copies and sequenced DGGE bands was higher at the later decay stages, which suggests that the importance of the diazotrophs increases during the decay. However, no correlation between the *nifH* copy

* Corresponding author. Natural Resources Institute Finland, Latokartanonkaari 9, FI-00790, Helsinki, Finland.
E-mail address: raisa.makipaa@luke.fi (R. Mäkipää).

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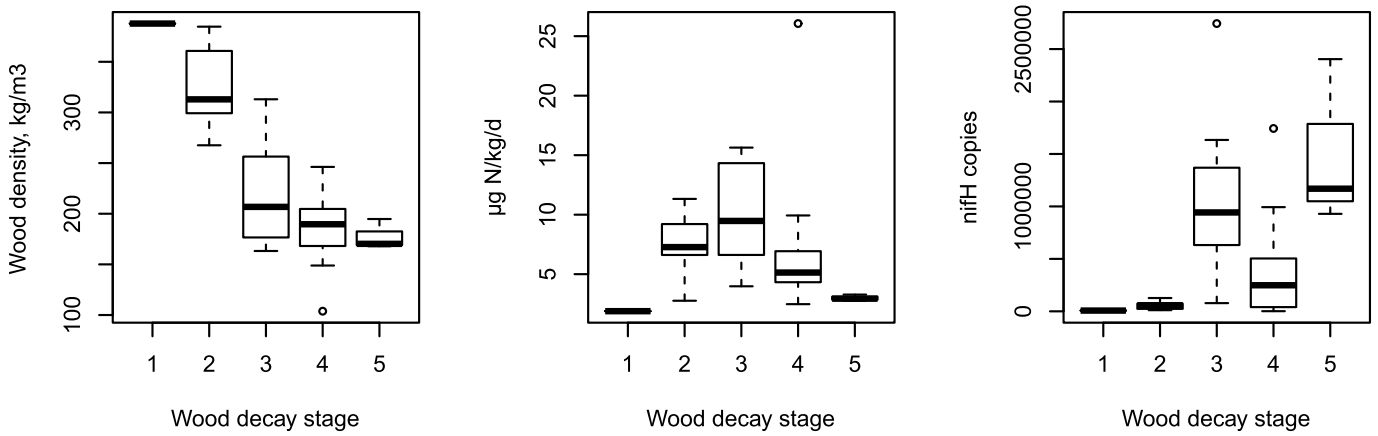

Fig. 1. Wood density (kg m^{-3}), nitrogen fixation (µg N kg^{-1} d^{-1}) and number of nifH gene copies (g^{-1} dry matter) varied according to wood decay stage ($p < 0.001$ for wood density (significant differences between decay classes (DC) 1 and 3–5; 2 and 3–5), $p = 0.053$ for sqrt of N fixation (significant difference between DC 3 and 5), and $p = 0.002$ for nifH copies (significant differences between DC 2 and 3; 2 and 5; 4 and 5)). Quartiles and whiskers with range of 1.5 are shown.

numbers and the N_2 fixation rate by ARA was found ($p = 0.249$). Neither was there a correlation between the N_2 fixation and the bacterial and the archaeal 16S rRNA gene copy numbers or the number of DGGE bands. The number of the bacterial copies correlated negatively with the wood density ($r = -0.49$, $p = 0.004$), but no correlation was found for the archaeal copies ($p = 0.8254$).

All recovered *nifH* sequences (representing 113 DGGE bands) from the decaying wood belonged to the Alphaproteobacteria, order Rhizobiales, and a major part (60%) of these were related to methane (CH_4) oxidizing bacteria (methanotrophs; Table 1). The methane oxidizing bacteria in this study represented the genera *Methylocapsa*, *Methyloferula*, and *Methylocystis*. These bacteria have been also found from forest soils and peatlands (Vorobev et al., 2011; Liebner and Svenning, 2013; Kolb, 2009). Hoppe et al. (2014) gained a similar result from a temperate forest dead wood, where their most abundant *nifH* sequence aligned with *Methyloferula stellata*, which was also one of the most abundant sequences together with *Methylocapsa acidiphila* in the present study. Furthermore, the 16S rRNA gene metagenome analysis conducted on decaying wood (Hoppe et al., 2015) supports these findings that methanotrophs have a role in the N_2-fixing process in decaying wood with high moisture content. Rinta-Kanto et al. (2016) showed that Alphaproteobacteria colonize N poor early decay stage wood, but they were also present in the N rich late decay wood. Actually, it is known that *nifH* is present in alphaproteo- and gammaproteobacterial (type I and II, respectively) methanotrophs (Auman et al., 2001). The ecological importance of methanotrophy in the N-cycle may be underestimated, since when using the conventional ARA method for measuring the N_2 fixation it dams the methane oxidation enzyme activity, and thus the activity of the methanotrophs is not easily detected (Flett et al., 1975) even though the method is used for this purpose (Auman et al., 2001). This may be the reason why no correlation between the *nifH* copy numbers and the N_2 fixation rate by ARA was found in our study. In a methanogenic *Sphagnum* dominated peatland ecosystem, the importance of methanotrophs in N_2 fixation was observed by using $^{15}N_2$ labelled N (Larmola et al., 2014), explaining over 30% of the peat N accumulation. Here, our results suggest that the methanotrophs also supply N to the decomposers in the N poor dead wood.

Hitherto, upland forests have not been classified as methanogenic environments, but recent findings of CH_4 emissions from shoots, tree trunks and canopies of Scots pine (Machacova et al., 2016; Halmeenmäki et al., 2017) show that we have to challenge this dogma even though methanogenic Archaea have not contributed to the process (Halmeenmäki et al., 2017). Furthermore, Lenhart et al. (2012) showed

Table 1
The closest matches for *nifH* nucleotide sequences. MOTU no. is the GenBank accession number for representative sequence of the molecular operational taxonomic unit derived from dead wood in this study, DC is occurrence in different decay stages (1–5), frequency is the number of similar sequences in the data set, organism is the closest matching bacterial species in the database and acc. no. refers to its accession number.

MOTU no.	DC	Frequency	Identity%	Organism	Acc. no.
KJ997919	1,2,3,4,5	15	97	*Methylocapsa acidiphila*	AM110721
KJ997918	2,3,4,5	13	99	*Methyloferula stellata*	FR686351
KJ997916	2,3,4,5	12	94	*Bradyrhizobium* sp.	FJ347439
KJ997926	3,4,5	11	87	*Bradyrhizobium* sp.	KM093817
KJ997922	3,4,5	9	85	*Methylocystis bryophila*	HE798545
KJ997923	3,4,	9	91	*Bradyrhizobium* sp.	FJ347433
KJ997925	3,4,5	7	98	*Methylocapsa acidiphila*	AM110721
KJ997928	3,4,5	7	92	*Azorhizobium caulinodans*	AM110700
KJ997932	2,4,5	6	85	*Methylocystis bryophila*	HE798545
KJ997917	2,3,4,5	5	93	*Methyloferula stellata*	FR686351
KJ997921	3,4	5	86	*Methylocystis* sp.	HE956757
KJ997927	3,5	3	93	*Bradyrhizobium diazoefficiens*	KX019788
KJ997935	2,4	3	97	*Methylocapsa acidiphila*	AM110721
KJ997931	3, 4	2	89	*Methylocystis bryophila*	HE798545
KJ997920	3	1	85	*Methylocystis bryophila*	HE798549
KJ997924	4	1	89	*Methylocystis bryophila*	HE798549
KJ997929	4	1	86	*Rhodopseudomonas palustris*	CP001096
KJ997930	2	1	96	*Bradyrhizobium* sp.	AM110702
KJ997933	2	1	86	*Bradyrhizobium* sp.	AB079616
KJ997934	2	1	98	*Methylocapsa acidiphila*	AM110721

that five different decomposer fungi produce variable amounts of CH_4 under aerobic conditions. As we have shown that methanotrophs are members of the nitrogen-fixing communities in all wood decay stages (Table 1), their abundance, community and contribution to the N-cycle ought to be studied using labeling techniques ($^{13}CH_4$ & $^{15}N_2$) and new deep-sequencing methods in order to fully understand the role of the diazotrophs-methanotrophs consortium in the C/N cycle as well as their role in the decay process.

Our result show that methanotrophs are members of the *nifH* community in dead wood, where decomposition is driven by fungi, leads to a hypothesis for further research: *Decomposer fungi that are capable of producing CH_4 feed methane-oxidizing bacteria to fix atmospheric N_2*. If this is the case, these fungi achieve a competitive advantage compared to other decomposers since N is the most important nutrient limiting the ecosystem processes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.02.012.

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