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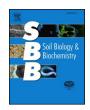
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Short Communication

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



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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 $52.9\,\mu g\,N\,k g^{-1}d^{-1}$. 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 ($60^{\circ}28'N$, $25^{\circ}12'E$). 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 $52.9\,\mu g\,N\,kg^{-1}\ d^{-1}$. The number of the \it{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 \it{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 \it{nifH} copy

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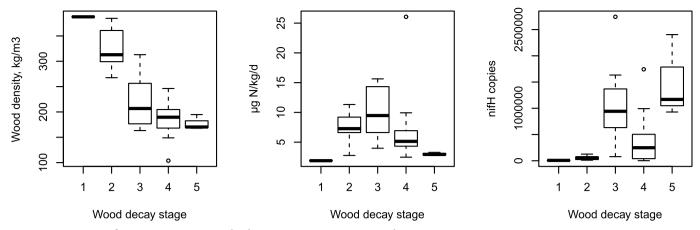


Fig. 1. Wood density (kg m $^{-3}$), nitrogen fixation (µg N kg $^{-1}$ d $^{-1}$) and number of nifH gene copies (g $^{-1}$ dry matter) varied according to wood decay stage (p < 0.001 for wood density (significant differences between decay classes (DC) 1 and 3–5; 2 and 3–5), p = 0.053 for sqrt of N 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 (CH₄) 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 N2-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 N2 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 N2 fixation rate by ARA was found in our study. In a methanogenic Sphagnum dominated peatland ecosystem, the importance of methanotrophs in N2 fixation was observed by using ¹⁵N₂ 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 CH₄ 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 ($^{13}\text{CH}_4 \ \& ^{15}\text{N}_2$) 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.

References

- Auman, A.J., Speake, C.C., Lidstrom, M.E., 2001. nifH sequences and nitrogen fixation in type I and type II methanotrophs. Applied and Environmental Microbiology 67, 4009–4016
- Bebber, D.P., Watkinson, S.C., Boddy, L., Darrah, P.R., 2011. Simulated nitrogen deposition affects wood decomposition by cord-forming fungi. Oecologia 167, 1177–1184.
- Brunner, A., Kimmins, J.P., 2003. Nitrogen fixation in coarse woody debris of *Thuja plicata* and *Tsuga heterophylla* forests on northern Vancouver Island. Canadian Journal of Forest Research 33, 1670–1682.
- DeLuca, T.H., Zackrisson, O., Nilsson, M.-C., Sellstedt, A., 2002. Quantifying nitrogenfixation in feather moss carpets of boreal forests. Nature 419, 917–920.
- Flett, R.J., Rudd, J.W.M., Hamilton, R.D., 1975. Acetylene reduction assays for nitrogen fixation in freshwaters: a note of caution. Applied Microbiology 29 (5), 580–583.
- Halmeenmäki, E., Heinonsalo, J., Putkinen, A., Santalahti, M., Fritze, H., Pihlatie, M.,
 2017. Above- and belowground fluxes of methane from boreal dwarf shrubs and *Pinus sylvestris* seedlings. Plant and Soil. http://dx.doi.org/10.1007/s11104-017-3406-7.
 Hicks, W.T., Harmon, M.E., Myrold, D.D., 2003. Substrate controls on nitrogen fixation

- and respiration in woody debris from the Pacific Northwest, USA. Forest Ecology and Management 176, 25–35.
- Hoppe, B., Kahl, T., Karasch, P., Wubet, T., Bauhus, J., Buscot, F., Krüger, D., 2014. Network analysis reveals ecological links between N-fixing bacteria and wood-decaying fungi. PLoS One 9, e88141.
- Hoppe, B., Purahong, W., Wubet, T., Kahl, T., Bauhus, J., Arnstadt, T., Hofrichter, M., Buscot, F., Krüger, D., 2015. Linking molecular deadwood-inhabiting fungal diversity and community dynamics to ecosystem functions and processes in Central European forests. Fungal Diversity 77, 367–379.
- Kolb, S., 2009. The quest for atmospheric methane oxidizers in forest soils. Environmental Microbiology Reports 1, 336–346.
- Larmola, T., Leppänen, S.M., Tuittila, E.S., Aarva, M., Merilä, P., Fritze, H., Tiirola, M., 2014. Mehanotrophy induces nitrogen fixation during peatland development. Proceedings of the National Academy of Sciences USA 111, 734–739.
- Lenhart, K., Bunge, M., Rathering, S., Neu, T.R., Schuttmann, I., Greule, M., Kammann, C., Schnell, S., Muller, C., Zorn, H., Keppler, F., 2012. Evidence for methane production by saprothrofic fungi. Nature Communications. http://dx.doi.org/10.1038/ ncomms2049.
- Leppänen, S.M., Salemaa, M., Smolander, A., Mäkipää, R., Tiirola, M., 2013. Nitrogen fixation and methanotrophy in forest mosses along a N deposition gradient. Environmental and Experimental Botany 90, 62–69.
- Liebner, S., Svenning, M.M., 2013. Environmental transcription of mmoX by methaneoxidizing Proteobacteria in a Subartic Palsa peatland. Applied and Environmental Microbiology 79, 701–706.
- Machacova, K., Bäck, J., Vanhatalo, A., Halmeenmäki, E., Kolari, P., Mammarella, I., Pumpanen, J., Acosta, M., Urban, O., Pihlatie, M., 2016. *Pinus sylvestris* as a missing source of nitrous oxide and methane in boreal forest. Scientific Reports 6, 23410. http://dx.doi.org/10.1038/srep23410.
- Poly, F., Monrozier, L.J., Bally, R., 2001. Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. Research in Microbiology 152, 95–103.
- Rajala, T., Peltoniemi, M., Pennanen, T., Mäkipää, R., 2012. Fungal community dynamics in relation to quality of decaying Norway spruce (*Picea abies* (L.) Karst.) logs in boreal forest. FEMS Microbiology Ecology 81, 494–505.
- Rinne, K.T., Rajala, T., Peltoniemi, K., Chen, J., Smolander, A., Mäkipää, R., 2017. Accumulation rates and sources of external nitrogen in decay wood in a Norway spruce dominated forest. Functional Ecology 31, 530–541. http://dx.doi.org/10. 1111/1365-2435.12734.
- Rinta-Kanto, J.M., Sinkko, H., Rajala, T., Al-Soud, W.A., Sørensen, S.J., Tamminen, M.V., Timonen, S., 2016. Natural decay process affects the abundance and community structure of bacteria and Archaea in *Picea abies* logs. FEMS Microbiology Ecology 92 (7). https://doi.org/10.1093/femsec/fiw087.
- Todd, R.L., Meyer, R.D., Waide, J.B., 1978. Nitrogen fixation in a deciduous forest in the South-Eastern United States. Ecological Bulletins 26, 172–177.
- Vitousek, P.M., Hobbie, S., 2000. Heterotrophic nitrogen fixation in decomposing litter: patterns and regulation. Ecology 81, 2366–2376.
- Vorobev, A.V., Baani, M., Doronina, N.V., Brady, A.L., Liesack, W., Dunfield, P.F., Dedysh, S.N., 2011. *Methyloferula stellate* gen. nov., sp. Nov., an acidophilic obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase. International Journal of Systematic and Evolutionary Microbiology 61, 2456–2463.
- Weisshaupt, P., Pritzkow, W., Noll, M., 2011. Nitrogen metabolism of wood decomposing basidiomycetes and their interaction with diazotrophs as revealed by IRMS. International Journal of Mass Spectrometry 307, 225–231.