

Bacteria inhabiting deadwood of 13 tree species are heterogeneously distributed between sapwood and heartwood

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Summary

Deadwood represents an important structural component of forest ecosystems, where it provides diverse niches for saproxylic biota. Although wood-inhabiting prokaryotes are involved in its degradation, knowledge about their diversity and the drivers of community structure is scarce. To explore the effect of deadwood substrate on microbial distribution, the present study focuses on the microbial communities of deadwood logs from 13 different tree species investigated using an amplicon based deep-sequencing analysis. Sapwood

and heartwood communities were analysed separately and linked to various relevant wood physico-chemical parameters. Overall, Proteobacteria, Acidobacteria and Actinobacteria represented the most dominant phyla. Microbial OTU richness and community structure differed significantly between tree species and between sapwood and heartwood. These differences were more pronounced for heartwood than for sapwood. The pH value and water content were the most important drivers in both wood compartments. Overall, investigating numerous tree species and two compartments provided a remarkably comprehensive view of microbial diversity in deadwood.

Introduction

Deadwood represents an important structural component of forest ecosystems and contributes to a number of ecosystem functions (Stokland *et al.*, 2012). The decay of deadwood is mediated by saproxylic organisms and influenced by a broad range of environmental determinants. Extrinsic factors such as temperature, moisture, general forest site properties or management strategies influence decay processes (Merganičová *et al.*, 2012). Intrinsic factors like the chemical and physical properties of the wood itself can also affect the decomposition process (Sorz and Hietz, 2006; Sjöström, 2013). In general, chemical composition differs greatly between coniferous and deciduous trees (Weedon *et al.*, 2009). The contents of poorly-degradable lignin, hemicelluloses and cellulose vary between tree species as well as between tree types (coniferous vs. deciduous wood). Low-molecular weight compounds, so-called extractives soluble in organic solvents, also differ between tree species, and their amounts are generally greater in conifers. These extractives include oils, waxes, resins, tannins and/or polyphenols, at least some of which are toxic and thus inhibit the growth of saproxylic organisms resulting in lower rates of decay (Kahl *et al.*, 2017). Even within a single log, physico-chemical properties vary spatially (Meerts, 2002). Heartwood, the inner part of the tree,

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has been separated from the living part of the tree for much longer than the outer sapwood which carries water, dioxygen and nutrients. The heartwood is usually harder and more resistant than the sapwood due to its higher lignin content, and to the presence of tannins and other biocidal substances. Thus, dioxygen availability, water content and/or pH can differ markedly between these two wood compartments (Simpson and TenWolde, 1999; Noll *et al.*, 2016).

The structural complexity of wood provides diverse environments for saproxylic biota and thus the wood-inhabiting communities may differ among tree species and probably between sapwood and heartwood (Seibold *et al.*, 2015; Baber *et al.*, 2016; Gossner *et al.*, 2016; Purahong *et al.*, 2018a). Although it remains largely unclear which determinants influence which group of organisms and to what extent, there is no doubt that deadwood represents a habitat for many specialized organisms and that it offers a range of niches for microbial life (fungi, bacteria, archaea and protists). Decaying wood is therefore a key factor for biodiversity in forest ecosystems and one that should be better understood in the interests of conservation strategies (Lachat *et al.*, 2013). It is well known that filamentous fungi contribute significantly to wood decay, in particular by initiating the breakdown of recalcitrant lignin and cell-wall polysaccharides (Hatakka and Hammel, 2010). The formation of visible sporocarps (fruiting bodies) has long facilitated the investigation of macro-fungi (Basidiomycota, Ascomycota) and their role in the decomposition process (Lachat *et al.*, 2013; Seibold *et al.*, 2015). The recent rapid advances made in molecular, culture-independent techniques have triggered an interest in non-visible fungal mycelia colonizing deadwood without necessarily forming fruit bodies. This has rapidly led to the first indications that many more, less obviously visible, fungi than previously suspected may contribute to decomposition processes (Kubartova *et al.*, 2012; Hoppe *et al.*, 2016; Purahong *et al.*, 2018a). This approach has also enabled further groups of microbes, for example, bacteria and archaea, to be taken into account without the need to face the associated challenges of culturing. The first studies on wood-inhabiting prokaryotes based on molecular sequence information have been performed within the last decade. In this context, Hoppe *et al.* (2015) were the first to intensively investigate bacterial community structure in natural deadwood of two different tree species, *Fagus sylvatica* and *Picea abies*, in relation to their respective wood physico-chemical properties. Other recent investigations in this area have substantially expanded our knowledge of prokaryotic diversity in the deadwood of other tree species and in other natural environments (Kielak *et al.*, 2016; Rinta-Kanto *et al.*, 2016; Tláškal *et al.*, 2017). To the best of our knowledge, spatial differentiation between

bacterial communities in sapwood and heartwood has been analysed only for the Asian conifer *Keteleeria evelyniana*, by Zhang *et al.* (2008), who interestingly observed higher diversity in the heartwood of this tree species.

It is well known that prokaryotes are responsible for several key ecosystem processes (chemolithotrophy, denitrification, nitrogen fixation, desulfurization, methylotrophy, methanogenesis, etc.). They thus contribute to carbon, nitrogen and sulfur cycling and render resources available for other biota that act as decomposers of a broad range of substrates (Lladó *et al.*, 2017). Due to their specialized enzymatic capabilities, some bacterial taxa are capable of degrading cellulose and hemicelluloses, and their contribution to lignin degradation (or its chemical modification) is currently under discussion (Johnston *et al.*, 2016). Hence, it is an important requirement that prokaryotes should be considered in forest biodiversity monitoring, in order to maximize our understanding of ecosystem functions. Despite this, exceedingly little is known about bacteria and especially archaea in deadwood environments compared with fungi (Johnston *et al.*, 2016).

The present work follows on from previous investigations using the same experimental platform – the BELongDead (Biodiversity Exploratories Long term Deadwood) experiment, which explores the process of decomposition in deadwood logs from 13 deciduous and coniferous temperate tree species with one starting point for decay (Bantle *et al.*, 2014a, b; Kahl *et al.*, 2015; Baber *et al.*, 2016; Noll *et al.*, 2016; Purahong *et al.*, 2018a, b). It has been shown that C, N and CO₂ release, enzyme activities and fungal diversity vary significantly between deadwood of different tree species, especially between deciduous and coniferous types.

In this recently-conducted study, we aimed at investigating the prokaryotic communities of these well-characterized deadwood logs by performing an amplicon based deep-sequencing analysis. Looking at this broad range of standardized deadwood tree species, which exhibit various wood physico-chemical properties, allows us to identify significant determinants of community structure and the main microbial drivers associated with the decomposition of deadwood. Furthermore, discriminating between sapwood and heartwood communities enables us to gain a comprehensive and detailed view of the spatial (radial) distribution of prokaryotes. In particular, we addressed the following questions:

1. Does the deadwood of nine deciduous and four coniferous tree species harbour different microbial communities?
2. Is there within-log variability between sapwood and heartwood?

3. Are tree species-related differences more pronounced in sapwood or in heartwood?
4. Which wood properties correspond to or determine the structure of deadwood-inhabiting microbes?

Results

Sequence data at a glance

After quality trimming and merging, a total of 2 646 021 sequences were used for bioinformatic analyses. A further 112 162 sequences (4.24%) that were identified as potential chimeras were removed from the data set. Clustering of the remaining sequences at a 97% similarity threshold revealed 12 655 OTUs (operational taxonomic units). The elimination of 1065 non-prokaryotic sequences (743 OTUs, often mitochondrial), 2864 singletons, 1274 doubletons and 746 tripletons resulted in a final list of 5965 OTUs based on 1 844 037 sequences. Taxonomic assignment was achieved for 5018 OTUs (84.1%) at the phylum level. Of these OTUs, 4592 (77.0%) were assigned to a class, 4041 (67.7%) to an order, 3517 (59.0%) to a family and 2858 OTUs (47.9%) down to the genus level. Based on sequence counts, 99.99% (1 843 940) could be classified to a phylum and 69.0% (1 272 641) to a genus. Overall, the domain of the Bacteria accounted for 98.5% of sequences and the domain of the Archaea for 1.5% of the entire data set. This corresponds to 5946 OTUs (99.7%) and 19 OTUs (0.3%) belonging to bacteria and archaea respectively.

Within the bacteria, Proteobacteria dominated at the phylum level, contributing 54.9% of all sequences. This was followed by Actinobacteria (14.2%), Acidobacteria (10.2%), Bacteroidetes (6.6%), Firmicutes (4.1%), Verrucomicrobia (1.9%) and Planctomycetes (1.3%). The most abundant bacterial classes in the entire data set turned out to be Alphaproteobacteria (24.9%), Gammaproteobacteria (14.3%), Betaproteobacteria (13.3%), Actinobacteria (10.8%), Acidobacteria (6.6%), Sphingobacteria (2.3%), Flavobacteriia (2.2%), Bacilli (1.9%), Thermoleophilia (1.8%), Clostridia (1.8%), Deltaproteobacteria (1.6%), Rubrobacteria (1.3%), Planctomycetia (1.3%) and Chitinophagia (1.1%).

Within the archaea, Euryarchaeota and Thaumarchaeota were the most abundant phyla accounting for 1.4% and 0.006% of all sequences respectively. Methanobacteria was the most dominant archaeal class, contributing for 1.2% of the entire data set.

Spatial community patterns

Microbial OTU richness ranged from 257 to 1418 with an average of 729 ± 288 (mean \pm SD). The mean OTU richness differed significantly between tree species, but not between deciduous and coniferous wood

(Fig. 1). Sapwood samples revealed significantly higher OTU richness than heartwood samples (797 ± 288 vs. 661 ± 275). Average values were highest for the tree species *Tilia* sp. (1054 ± 397) and lowest for *Pinus sylvestris* (417 ± 111).

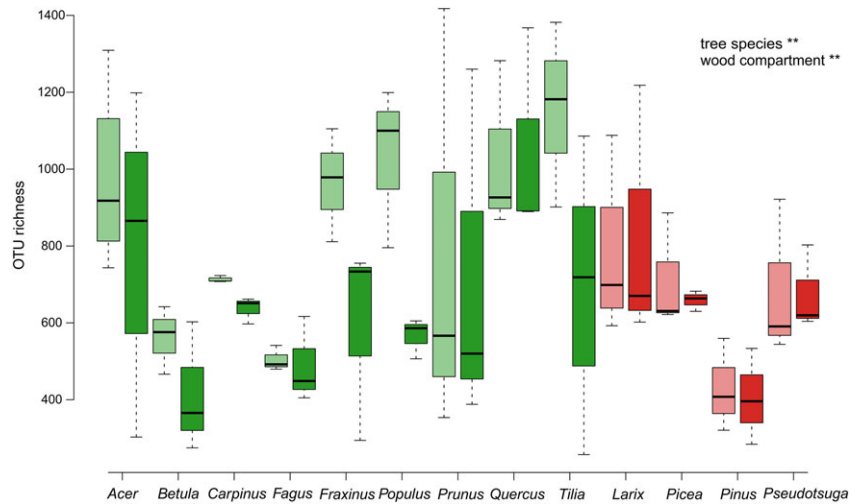
The relative abundances of all shown bacterial groups (except Betaproteobacteria) differed significantly among tree species and/or between sapwood and heartwood (Fig. 2). For instance, the most abundant proteobacterial class, the Alphaproteobacteria, both differed in abundance between tree species ($p < 0.05$) and were significantly more present in sapwood samples ($p < 0.001$). Acidobacteria were also highly variable between tree species ($p < 0.001$) and more frequently detected in sapwood ($p < 0.001$). This finding was in contrast to Gammaproteobacteria, which were more abundant in heartwood ($p < 0.001$). Firmicutes were detected specifically in the heartwood of the deciduous trees *Tilia* sp., *Betula* sp. and *Populus* sp.. Generally, greater variability was observed within heartwood communities. In *Populus* heartwood a high proportion of sequences belonged to the category 'others'. This is due to the fact that about 28% of sequences were affiliated to the archaeal phylum Euryarchaeota (Supporting Information Table S1) which only accounted for 1.4% of the entire data set and were therefore not considered in Fig. 2.

Permutational Multivariate Analysis of Variance (perMANOVA) applied to microbial community structure revealed significant differences between deciduous and coniferous trees (tree type), and among tree species, as well as between sapwood and heartwood (Table 1). NMDS ordinations separately performed for both wood compartments showed that this difference was more pronounced for heartwood communities than for communities in sapwood (Fig. 3A and B).

Microbial community structure associated with wood physico-chemical properties

Wood physico-chemical properties were found to correlate significantly with microbial community structure (Fig. 3A and B). Specifically, pH value, water content and the content of Klason and acid-soluble lignin contributed significantly to the variation observed, while C/N ratio and organic extractives did not (Table 2). Microbial communities of coniferous deadwood tree species clustered together, even when there was not complete separation from the communities of deciduous species. Microbial communities of *Tilia* sp., *Fraxinus excelsior* and *Populus* sp. were distinctly separated from those of all other tree species on the first NMDS axis, consistent with the relatively high pH values of these deciduous trees (Table 3). Communities detected in *Betula*

Fig. 1. Microbial OTU richness for sapwood and heartwood samples of each deadwood tree species. Statistical significance is based on three-way ANOVA related to tree species, tree type and wood compartment. Green = deciduous, red = coniferous, light shaded = sapwood, dark shaded = heartwood. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.



sp. showed clear separation from the other communities on the second NMDS axis, especially for the heartwood (Fig. 3B). This was correlated with the high water content observed for these logs (Table 3).

Ordination of the 100 most abundant OTUs according to their position in the sapwood and heartwood ordination demonstrated that communities were highly variable (Fig. 3C and D; Supporting Information Tables S2 and S3). Examining the mean relative abundances, indicated by circle sizes, the picture clearly shows that there were different distributions of these OTUs within sapwood and heartwood, and it also illustrates more distinct separation within heartwood.

Discussion

Microbial distribution related to tree species

Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Acidobacteria and Actinobacteria were among the most dominant bacterial phyla and proteobacterial classes. This is in line with previous investigations demonstrating that members of these phyla (comprising a range of chemoorganotrophic, facultatively anaerobic species) are prominent bacterial representatives in and on deadwood (Zhang *et al.*, 2008; Hoppe *et al.*, 2015; Kielak *et al.*, 2016; Tláškal *et al.*, 2017). However, even at this taxonomic level, relative sequence abundances were quite variable among tree

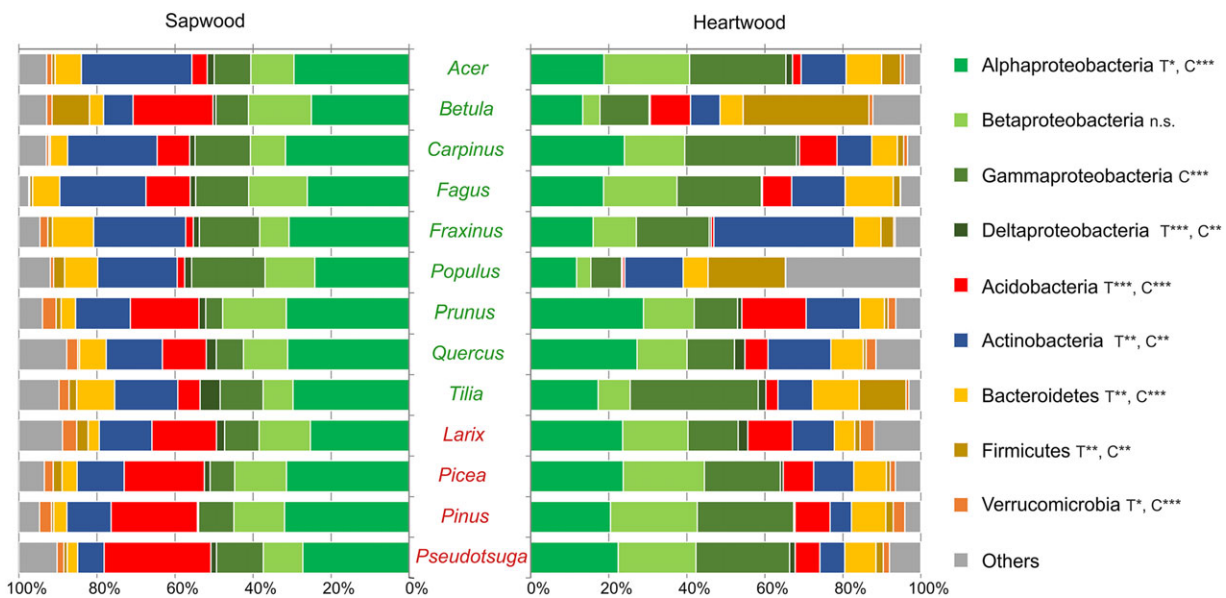


Fig. 2. Relative abundances of phylogenetic groups (bacterial phyla and proteobacterial classes) in deadwood from 13 different tree species (green = deciduous, red = coniferous). The category ‘others’ includes all prokaryotic phyla with < 1.5% relative sequence abundance and sequences that could not be taxonomically assigned to a prokaryotic phylum. Statistical significances are based on two-way ANOVA related to tree species (T) and wood compartment (C). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

species. This variation between tree species was also observed for OTU richness and community structure. NMDS analyses demonstrated that the microbial communities of coniferous logs clustered together, but we did not observe any clear differentiation from all deciduous species, as previously demonstrated for fungal sporocarps on the same deadwood logs (Baber *et al.*, 2016). This may be due to the fact, that the microbial communities of the nine investigated deciduous tree species were more variable and thus did not cluster as tightly as the communities from conifers. Other abiotic and biotic factors corresponding to different wood types must therefore play a prominent role in shaping the microbial community. Distinct distribution of bacteria on different tree species was reported by Hoppe *et al.* (2015), but this is not consistent with the findings of Tláskal *et al.* (2017), who did not observe significantly different bacterial communities on *Abies alba*, *Picea abies* and *Fagus sylvatica*. These contrasting observations may reflect the fact that we are still in the early stages of unravelling uncertainties about bacterial diversity and its determinants in deadwood.

Microbial distribution related to sapwood and heartwood

Microbial community structure and OTU richness differed significantly between the sapwood and the heartwood of the deadwood tree species investigated. OTU richness was higher in sapwood than in heartwood. Despite the contradictory results of Zhang *et al.* (2008), the higher number of OTUs in the outer part of the wood is not surprising. Although the mechanism of wood colonization by bacteria is largely unknown, four different origins are conceivable: (i) other wood-colonizing biota, especially fungal hyphae that actively invade the wood and function as highways for bacteria (Harms *et al.*, 2011), (ii) the air including airborne bacteria and their spores, (iii) the underlying soil and (iv) the wood itself (Johnston *et al.*, 2016). Hence, the highly diverse environment of the logs, including leaf-litter and soil (Purahong *et al.*, 2016), which can be regarded as a reservoir of potential wood-

colonizing biota, makes a higher level of microbial OTU richness in the outer sapwood quite plausible. Moreover, acid-soluble lignin, C/N ratio, water content and pH varied significantly between the two wood compartments, indicating that they provide different microclimates for microbial life. Heartwood often contains inhibitory and toxic extractives (Taylor *et al.*, 2002). Although this fact does not explain the community structure identified in the present study, it corresponds quite well with the observation of lower OTU richness in heartwood. Particularly, this reduced richness was detectable in deciduous logs, while this phenomenon was not as clearly pronounced in coniferous logs.

Tree species effect for sapwood and heartwood

Surprisingly, differences between tree species were more pronounced for heartwood communities than for the corresponding sapwood communities, as shown by their ordination on the first NMDS axes. The significance of these results is difficult to assess, as – to our knowledge – there has been no other study that compared heartwood communities among different tree species. Our finding raises a question about the origin of this ‘hidden’ microbial diversity. One can speculate that prokaryotes occurring mainly in heartwood may originate from the wood itself, and are thus not significantly involved in wood decomposition; rather, they represent ‘delayed’ prokaryotic endophytes that proliferate after tree death at the expense of easily assimilable wood constituents. However, since all deadwood logs in this artificial set-up originate from the same geographical area (the German Federal state of Thuringia), they have had a similar history and faced comparable external conditions and extrinsic factors influencing initial microbial colonization. The ‘endophyte question’ cannot, however, satisfactorily be answered here and it requires further investigation, as very little is known about prokaryotic tree endophytes (Johnston *et al.*, 2016). Nevertheless, the inhibitory substances of the heartwood likely differ between tree species. Thus, this wood compartment may require specialized

Table 1. PerMANOVA results, based on Bray–Curtis dissimilarities, for microbial community structure in relation to tree type (deciduous vs. coniferous logs), tree species and wood compartment (sapwood vs. heartwood)

	Df	Sum Sq	Pseudo <i>F</i>	<i>R</i> ²	<i>p</i>
Tree type	1	1.8303	9.4633	0.07872	0.001***
Tree species	11	6.4225	3.0188	0.27623	0.001***
Wood compartment	1	1.7833	9.2203	0.07670	0.001***
Tree type × wood compartment	1	0.1552	0.8023	0.00667	0.632
Tree species × wood compartment	11	2.2281	1.0473	0.09583	0.314
Residuals	56	10.8309		0.46584	
Total	81	23.2503		1.00000	

Df, degrees of freedom; Sum Sq, sum of squares; Pseudo-*F*, *F* value by permutation; significant factors are indicated in bold with *p* < 0.05, *p*-values based on 999 permutations (lowest *p*-value possible 0.001).

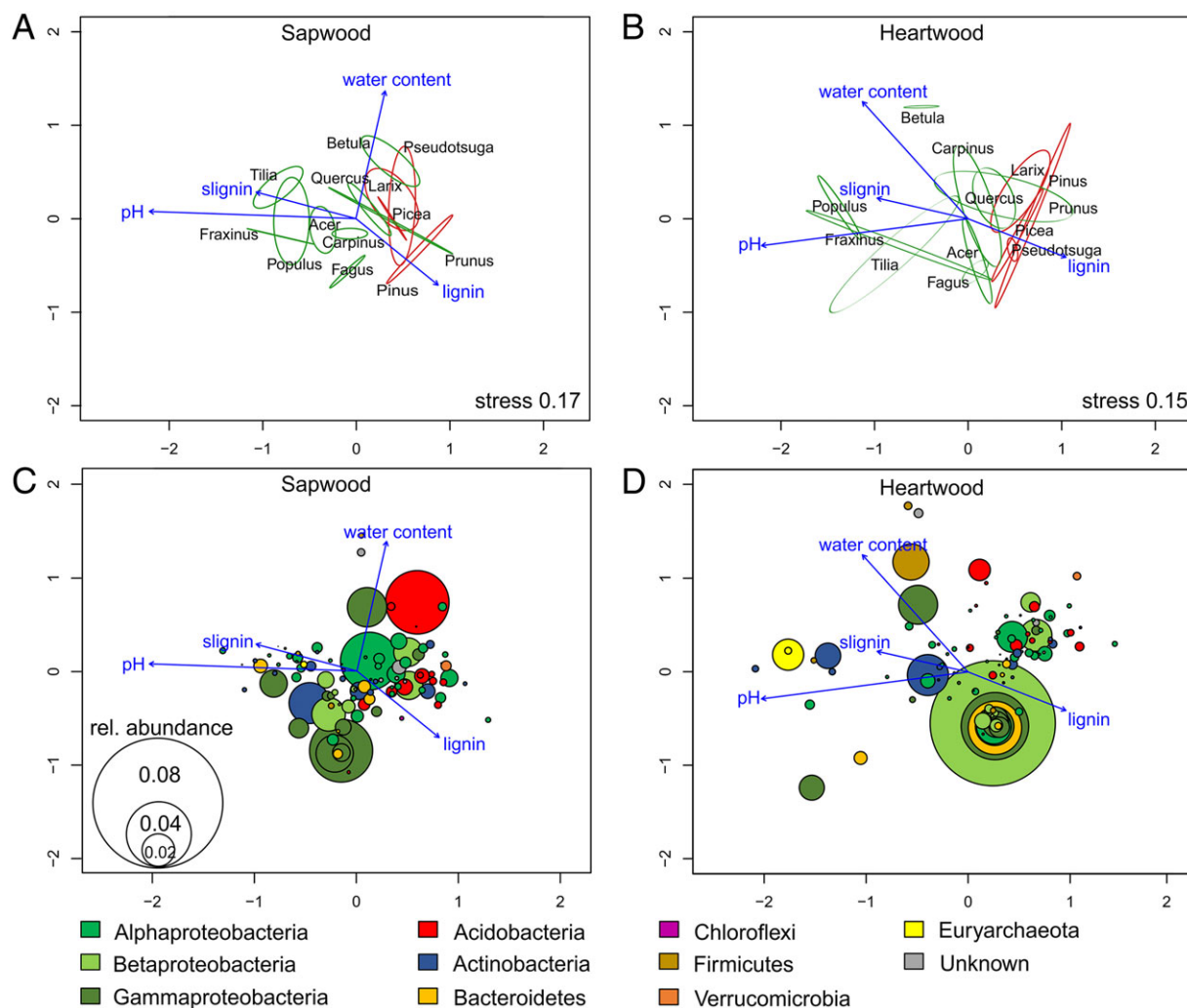


Fig. 3. Non-metric multidimensional scaling (NMS) ordination displaying microbial community structure for (A) sapwood and (B) heartwood in relation to tree species (green = deciduous, red = coniferous), pH value, water content, Klason lignin and acid-soluble lignin (slignin). Extractives and the C/N ratio, which were non-significant factors, are not shown. (C) and (D). Bubble charts displaying the distribution of the 100 OTUs most abundant in the entire dataset, their averaged abundances (circle size) and colour codes indicating their prokaryotic phyla or proteobacterial classes (full list Table S2 and S3).

characteristics from the inhabiting microbes resulting in more difference between microbial communities in heartwood than in sapwood. Hence, the high tree species-related microbial variability and the lower number of OTUs in the heartwood of several of the investigated logs indicates that this compartment may constitute a harsh environment that offers an appropriate habitat for only a small number of well adapted microbes.

Wood properties related to the structure of deadwood-inhabiting prokaryotes and the main microbial drivers corresponding to the variations observed

Even where the communities of the sapwood and the heartwood differed, their structure was explained by the same physical and chemical properties of the wood. The pH value was found to be the most important driver of

microbial community structure. In particular, communities of trees with high pH, such as *Populus* sp., *Fraxinus* sp. and *Tilia* sp., were clearly separated from logs with

Table 2. Goodness-of-fit statistics (R^2), based on the 'envfit' function, for wood parameters fitted to the two-dimensional non-metric multidimensional scaling (NMS) ordination of microbial community structure

Parameter	Sapwood		Heartwood	
	R^2	p	R^2	p
Klason lignin	0.1620	0.045	0.1793	0.021
Acid-soluble lignin	0.1544	0.050	0.1412	0.048
Water content	0.2487	0.002	0.4046	0.001
pH	0.6185	0.001	0.7046	0.001
Extractives	0.0457	0.399	0.0816	0.196
C/N	0.0460	0.408	0.0949	0.152

Significance estimates were based on 999 permutations. Significant factors are indicated in bold.

Table 3. Physico-chemical parameters in deadwood of 13 different tree species ($n = 3$, Mean \pm SD); modified from Noll et al. (2016)

Tree species	Klason lignin [%]		Acid-soluble lignin [%]		C/N		Water content [g g ⁻¹ dry mass]		pH		Extractives [%]	
	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood
Deciduous												
<i>Acer</i> ¹	25.9 \pm 0.7	25.1 \pm 0.6	2.3 \pm 0.1	2.6 \pm 0.3	227 \pm 111	370 \pm 21	0.5 \pm 0.1	0.5 \pm 0.2	4.4 \pm 0.3	4.4 \pm 0.2	1.3 \pm 0.3	1.3 \pm 0.1
<i>Betula</i>	20.3 \pm 2.6	20.6 \pm 1.5	3.3 \pm 0.3	3.3 \pm 0.1	259 \pm 132	389 \pm 52	1.5 \pm 0.7	1.2 \pm 0.3	4.4 \pm 0.3	5.0 \pm 0.1	1.5 \pm 0.9	1.8 \pm 0.5
<i>Carpinus</i>	18.7 \pm 1.6	18.0 \pm 1.9	3.7 \pm 0.1	3.8 \pm 0.1	307 \pm 131	331 \pm 200	0.3 \pm 0.2	0.6 \pm 0.1	4.3 \pm 0.1	4.8 \pm 0.6	1.4 \pm 0.3	1.1 \pm 0.3
<i>Fagus</i>	24.8 \pm 1.7	24.3 \pm 0.6	2.5 \pm 0.2	2.7 \pm 0.2	290 \pm 151	439 \pm 59	0.2 \pm 0.0	0.5 \pm 0.1	5.2 \pm 0.3	5.2 \pm 0.8	1.0 \pm 0.2	0.9 \pm 0.1
<i>Fraxinus</i>	24.7 \pm 1.3	22.9 \pm 2.0	2.2 \pm 0.2	3.2 \pm 0.5	371 \pm 90	357 \pm 71	0.4 \pm 0.1	0.4 \pm 0.1	5.9 \pm 0.5	6.2 \pm 0.5	1.1 \pm 0.3	1.4 \pm 0.1
<i>Populus</i>	22.1 \pm 1.4	24.7 \pm 1.7	2.0 \pm 0.1	2.0 \pm 0.1	480 \pm 78	555 \pm 252	0.6 \pm 0.1	0.7 \pm 0.1	5.3 \pm 0.5	7.3 \pm 0.3	1.6 \pm 0.3	1.1 \pm 0.1
<i>Prunus</i>	29.6 \pm 9.7	27.3 \pm 7.2	2.8 \pm 0.3	2.8 \pm 0.4	278 \pm 50	486 \pm 82	1.5 \pm 1.1	0.7 \pm 0.1	4.3 \pm 0.8	4.1 \pm 0.9	3.0 \pm 1.8	3.5 \pm 2.1
<i>Quercus</i>	24.3 \pm 1.2	21.8 \pm 0.8	3.4 \pm 0.1	4.0 \pm 0.3	263 \pm 124	410 \pm 132	1.2 \pm 0.5	0.6 \pm 0.1	4.1 \pm 0.1	4.0 \pm 0.2	2.4 \pm 0.8	2.1 \pm 0.6
<i>Tilia</i>	22.6 \pm 9.2	20.5 \pm 5.1	3.4 \pm 0.4	3.6 \pm 0.1	298 \pm 67	387 \pm 27	1.0 \pm 0.4	0.8 \pm 0.3	5.9 \pm 1.3	5.8 \pm 0.9	2.6 \pm 0.1	3.1 \pm 1.1
Coniferous												
<i>Larix</i> ¹	29.7 \pm 1.2	28.0 \pm 0.8	0.6 \pm 0.1	0.4 \pm 0.0	557 \pm 142	604 \pm 205	0.9 \pm 0.5	0.4 \pm 0.0	4.5 \pm 0.5	4.5 \pm 0.3	2.0 \pm 1.3	2.3 \pm 0.7
<i>Picea</i>	32.7 \pm 7.3	28.7 \pm 2.2	0.6 \pm 0.2	0.5 \pm 0.1	294 \pm 283	516 \pm 198	0.9 \pm 0.4	0.4 \pm 0.0	3.8 \pm 0.5	4.4 \pm 1.0	2.4 \pm 2.2	1.2 \pm 0.3
<i>Pinus</i>	26.6 \pm 1.5	29.2 \pm 1.5	0.9 \pm 0.5	0.5 \pm 0.1	318 \pm 51	665 \pm 89	0.8 \pm 0.6	0.4 \pm 0.0	3.9 \pm 0.4	4.6 \pm 0.2	2.2 \pm 2.2	4.1 \pm 1.5
<i>Pseudotsuga</i>	28.0 \pm 1.9	29.7 \pm 0.6	0.5 \pm 0.2	0.4 \pm 0.1	427 \pm 210	576 \pm 65	1.0 \pm 0.5	0.4 \pm 0.0	4.1 \pm 0.3	4.4 \pm 0.1	1.6 \pm 0.5	3.5 \pm 1.7
Statistics	T***	T***	T***, C*, T	T***, C***	T***, C***	T***, C***	T***, C*, T	T***, C***	T***, C***	T***, C***	T***	T***

Statistical significances are based on two-way ANOVA applied to tree species (T) and wood compartment (C) for all parameters investigated. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; T^{***}, C^{***}, T^{***} × C^{***}.

low pH values, especially those of conifers. It is well known that the majority of bacteria are strongly affected by this factor, in particular by low and fluctuating pH and generally more affected than fungi (Rousk *et al.*, 2010; Nacke *et al.*, 2016). However, Hoppe *et al.* (2015) observed only a moderate effect, but explained it on the basis of the narrow range of pH (4.0–5.3) in deadwood logs of *Picea abies* and *Fagus sylvestris*, whereas the pH in the present study ranged from 3.8 (*Picea abies* sapwood) to 7.3 (*Populus* sp. heartwood).

Interestingly, the bubble chart clearly indicates that the presence of two archaeal OTUs was correlated with high pH. Although we did not specifically aim to assess archaeal diversity, the deep-sequencing approach identified 19 archaeal OTUs (1.5% of the entire data set), and two of them were among the 100 most abundant sequences. Even less is known about archaea in deadwood compared with bacteria. Recently, Thaumarchaeota have been found to be the dominant archaeal phylum in *Picea abies* logs, accounting for 0.4% of the total prokaryotic population, whereas the proportion of methanogenic Euryarchaeota was negligibly low (at 0.0003%) (Rinta-Kanto *et al.*, 2016). In soils, archaea have been observed to make up between 0.01% and 6.7% of the microbiome, with Thaumarchaeota being the dominant group (Fierer *et al.*, 2012). In the present study, *Populus* heartwood communities contained 28% euryarchaeal sequences on average, 80% of which were affiliated to the genus *Methanobacterium*. This methanogenic genus is reliant on pH-neutral, anaerobic and humid conditions (Bonin and Boone, 2006). The high sequence abundance of methanogenic archaea in all *Populus* heartwood samples means either that they were already present in the living tree or that they were rapidly introduced into the fresh dead heartwood of *Populus*, which may provide ideal conditions for their growth. This finding is consistent with the parallel occurrence of Firmicutes, the second most common group in *Populus* heartwood where it contributes approximately 20% of the sequences. This phylum comprises fermentative and homoacetogenic bacteria (Clostridiales) that form – always together with methanogenic archaea – syntrophic communities in specific environments, for example in sediments, digestion towers, the rumens of ruminant animals or insect guts (Morris *et al.*, 2013). In the latter context, the possibility therefore cannot be ruled out that xylophagous insects (e.g., beetle larvae; Hackstein, 2010) may have contributed to the high abundances of both microbial groups in the *Populus* logs. Only repeated future sampling, also including natural *Populus* deadwood from near the experimental set-up, will enable us to understand this isolated phenomenon.

While methanogenic archaea and fermentative Firmicutes thrive in *Populus* heartwood with its neutral pH,

other microbial groups may be inhibited under these conditions. For instance, among the 100 most abundant OTUs, Acidobacteria were present only at low pH (Fig. 3B and C), demonstrating that pH exerts a considerable influence on this group. Probably for this reason, in the heartwood communities of *Populus* sp. and *Fraxinus* sp., this otherwise dominant group accounted for less than 1% of the entire community. All these results clearly indicate that the growth of wood-inhabiting prokaryotes is affected by pH, as is also the case for forest soils (Lauber *et al.*, 2009; Nacke *et al.*, 2011).

Water content was the second most important driver of community structure in both wood compartments, but its effect was more pronounced in the case of heartwood. OTUs affiliated to the phylum Firmicutes were mainly responsible for the separation on the second NMDS axis that split *Betula* sp., the deadwood with highest water content, off from all other deadwood tree species. The closest matches for these OTUs were *Lactovum* and *Ruminiclostridium*. *Lactovum*, of which there is only one described species (*L. miscens*), is an anaerobic, aerotolerant bacterium (Lactobacillales, lactic acid bacteria) first isolated from forest soils, which is able to ferment cellobiose (Drake, 2014). *Ruminiclostridium* spp. are obligatory anaerobic, mesophilic bacteria that can hydrolyze cellulose and use the sugars released as carbon and energy sources in fermentative metabolism (Yutin and Galperin, 2013). Hence, both genera could play a role in wood decay under anaerobic conditions, as a high water content as we observed is often associated with decreasing dioxygen (O₂) availability (Kazemi *et al.*, 2001). In other studies, members of the phylum Firmicutes have been described as being moderately dominant, accounting for less than 10% of relative sequence abundance (Hoppe *et al.*, 2015; Tláskal *et al.*, 2017). Due to the spatially differentiated investigation here, this phylum can be seen to be of greater importance with regard to bacteria that are specialized in colonizing the heartwood of certain deadwood logs such as those of *Tilia* sp., *Populus* sp. and *Betula* sp., where they make up, respectively, 12%, 20% and 32% of all sequences. As bacteria seem to benefit from high wood moisture (Hu *et al.*, 2017), it can be anticipated that they will make a contribution to decomposition under the conditions of this experiment.

Although none of the dominant OTUs was clearly associated with lignin content, the community structure was significantly affected by this highly recalcitrant wood component, which acts as a molecular glue in the secondary cell-wall network. This effect was only weakly pronounced (goodness-of-fit statistics: $R^2 < 0.18$ in all cases) and equally pronounced for sapwood and heartwood. Klason lignin corresponded with the communities of coniferous deadwood tree species and acid-soluble lignin with those of deciduous trees. How and to what

extent bacteria are involved in the degradation of lignin cannot be answered here, but our results show that this wood component has at least some effect on them.

In contrast to our expectations, the content of organic extractives and the C/N ratio did not play any role in shaping communities, although these factors differed significantly between sapwood and heartwood and/or among tree species (Table 3). While the carbon and nitrogen contents or the C/N ratio have been considered in other microbial diversity studies and have been shown either to affect communities or not to do so (Hoppe *et al.*, 2015; Kielak *et al.*, 2016; Rinta-Kanto *et al.*, 2016; Tláskal *et al.*, 2017), the effect of organic extractives on microbial communities is largely unknown, with the exception of some initial insights given by Rinta-Kanto *et al.* (2016). Clearly, 'autochthonous' wood prokaryotes have to deal with varying amounts of this complex, inhibitory component (comprising resin terpenes, phenolics, waxes, quinones, etc.) in each particular tree species, and this is generally of more importance in conifers, resulting in lower decay rates (Table 3), (Noll *et al.*, 2016; Kahl *et al.*, 2017).

Conclusions

Microbial communities are affected by several environmental factors, but not all wood-structure components influence these communities. There are several reports stating that dioxygen availability, directly or indirectly depending on water content and pH, and the latter in their own right, are critical factors affecting wood-inhabiting biota and thus the decomposition rate (Kazemi *et al.*, 2001; Cornelissen *et al.*, 2012; Johnston *et al.*, 2016). These findings have been confirmed by our results. However, as wood decay mediated by fungi leads to the acidification of tree logs (due to the secretion of organic acids), further investigations into the patterns of co-occurrence of fungi and prokaryotes are necessary in order to evaluate whether the pH value determines their communities or whether fungal activity (and thus the presence of fungi) structures microbial communities by modifying pH. Ongoing investigations using the BELongDead experimental platform over the next years will allow us to address this problem better and to fill existing research gaps. For instance, the succession in microbial diversity should be considered, since wood quality changes greatly as a result of the decay process (Rajala *et al.*, 2012; Hoppe *et al.*, 2016). Moreover, in addition to the appropriate patterns of co-occurrence of fungi and prokaryotes, their interaction with saproxylic beetles is also of importance if ecosystem processes in decaying wood are to be understood, not least with respect to the presence of methanogenic archaea. It is noteworthy that to date, the relationships of these

saproxyllic biota (insects, bacteria, archaea, fungi) have not been investigated in a holistic sense.

We are aware that DNA based community analyses do not allow drawing conclusions on the actual rate of microbial colonization and on their contribution to decomposition. Therefore, comparisons of relative abundances only give a partial view on the ecology and spatial distribution of microbial communities. Nonetheless, the present study provides a remarkably comprehensive insight into the microbial diversity in deadwood, revealing a high degree of spatial heterogeneity between sapwood and heartwood and thus extending the conclusion of Kahl *et al.* (2017) that a mix of different deadwood tree species may be most effective in promoting species diversity in the case of prokaryotes. Moreover, due to the wide range of physico-chemical properties of the wood making up the selected deadwood logs, our findings help us to gain a better understanding of the environmental factors corresponding to microbial communities in decaying wood.

Experimental procedures

Study area and sampling

The study was performed on forest plots of the German Biodiversity Exploratories located at the Nationalpark Hainich in Central Germany (N 51.08, E 10.43) (Fischer *et al.*, 2010). In late 2008 an experimental platform for researching deadwood decomposition was established, and named the BELongDead experiment. The experimental design was introduced in more detail in Kahl *et al.* (2017). Briefly, freshly cut logs of 13 temperate tree species [9 deciduous species: maple (*Acer* sp., Sapindaceae), birch (*Betula* sp., Betulaceae), hornbeam (*Carpinus* sp., Betulaceae), beech (*Fagus sylvatica* L., Fagaceae), ash (*Fraxinus excelsior* L., Oleaceae), aspen (*Populus* spp., Salicaceae), wild cherry (*Prunus avium*, Rosaceae), oak (*Quercus* spp., Fagaceae) and lime tree (*Tilia* spp., Malvaceae) and 4 coniferous species: larch (*Larix decidua* Mill., Pinaceae), Norway spruce (*Picea abies* L., H. Karst., Pinaceae), pine (*Pinus sylvestris* L., Pinaceae) and Douglas fir (*Pseudotsuga menziesii* (Mirb.), Franco, Pinaceae)] were placed, using three replicates of each, in representative research plots to investigate their decomposition over the long term. In June 2014, sampling of wood chips of a subset of 13 deadwood logs on each of three experimental plots was carried out as described in detail in Noll *et al.* (2016). Two additional logs of *Acer* sp. and *Larix decidua* were sampled as they were incorrectly placed in the initial experimental set-up in 2008. Distinguishable sapwood and heartwood samples were collected in the form of chips by driving an auger horizontally to the centre (15–20 cm deep) of each of the selected

logs. After bark removal, sapwood was collected by means of an initial drilling (the first 5 cm of depth) followed by a second drilling to collect heartwood. To avoid contamination, the auger was flamed between each deadwood sampling and the next. A total of 82 (13 tree species × 3 experimental plots × 2 sampling depths, + sapwood and heartwood from 2 additional logs) samples was processed and analyzed.

DNA extraction, PCR and sequencing

Total community DNA was isolated from 0.25 g of each homogenized wood sample using a ZR Soil Microbe DNA MiniPrep kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. The quantity of genomic DNA was checked using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Dreieich, Germany). The prokaryotic 16S region was amplified using a modified primer mix: P5_8N_515F and P5_7N_515F (forward) together with P7_2N_806R + P7_1N_806R (reverse, compare Supporting Information Table S4), according to (Caporaso *et al.*, 2012). PCR was performed in 25 µl triplicate reactions, containing 12.5 µl of GoTaq Green Mastermix (Promega, Madison), 25 µM of each primer and approximately 20 ng template DNA. Cycling conditions were as follows: a denaturation period of 3 min at 94°C followed by 32 cycles of 94°C for 45 s, 50°C for 1 min, 72°C for 1 min 30 s and a final elongation step at 72°C for 10 min. After checking the quality of the PCR products by separation on a 1.5% agarose gel, the replicates were pooled and purified by gel extraction using an innuPREP Gel Extraction Kit (Analytik Jena, Jena, Germany). The purified DNA was quantified using a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies, Waldbronn, Germany). Subsequently the PCR products were sequenced with an Illumina MiSeq at the Deep Sequencing Group of the Technical University Dresden. Briefly, purified PCR products with universal 5' tails were subjected to a second PCR of 6–8 cycles using Phusion HF (NEB, Ipswich, MA) and two indexing primers, the P5 and the P7 primer (Supporting Information Table S4). After indexing PCR, the final libraries were purified (1× Agencourt AMPure XP Beads, Beckman Coulter, Krefeld, Deutschland), pooled in equimolar amounts and used for 2 × 300 bp paired end sequencing on a MiSeq System from Illumina.

Bioinformatics

Raw sequence data were imported and processed using Geneious R9 (Kearse *et al.*, 2012). First, all forward and reverse reads were 5' trimmed and adapter regions excluded. Then forward and reverse reads were paired, and samples were separated ('demultiplexed') based on

specific primer regions and indices followed by exclusion of primer sequences. Paired prokaryotic sequences were quality trimmed using BBDuk (settings: trim low quality, minimum quality = 13) and merged to obtain the full length V4 region of the 16S rRNA genes using BBMerge (merge rate settings: very high) from BBTools. Generated sequences of 160–320 bp length were exported, for clustering and OTU table generation, into SEED 2.0.4 (Vetrovsky and Baldrian, 2013). Clustering was performed using USEARCH 8.1.1861 (32 bit) and included chimera removal. OTU separation was based on 3% sequence dissimilarity. OTU tables were generated and taxonomy was inferred by blast search against the nr dataset (Vetrovsky and Baldrian, 2013). OTUs that comprised only singletons, doubletons and tripletons were not subjected to further analyses. All processed and merged sequences have been submitted to the NCBI short read archive (SRA, <https://www.ncbi.nlm.nih.gov/sra/>) and are accessible under the number SRP102646.

Wood parameters

Analyses of wood parameters and related procedures were described previously in Noll *et al.* (2016) and Arnstadt *et al.* (2016). Briefly, all frozen samples were ground with an analytical mill (IKA A11 basic, IKA-Werke GmbH & Co.KG, Staufen, Germany) and 3 g of wood was extracted with 30 ml distilled water by shaking on a Certomat MO II rotary shaker (Sartorius AG, Göttingen, Germany) at 4°C and 120 rpm for 120 min. The aqueous extracts were filtered through a Steriflip filter unit (nylon net, 100 µm, Merck Millipore, Schwalbach, Germany) and centrifuged for 10 min at 16 000 g and 10°C (centrifuge 5424R, Eppendorf, Germany). Aqueous extracts were used to determine pH, water content, C and N, organic extractives, Klason lignin and acid-soluble lignin.

For C and N analysis, all milled wood samples were freeze dried and measured using an elemental analyzer according to the manufacturer's protocol (NA 1108 CE instruments, Milan, Italy). The water-extracted woody material was then dried at 60°C and applied to an accelerated solvent extraction device (Dionex ASE 200) with acetone (cell size, 5 ml; preheating 1 min; static equilibration period 100°C, 1500 PSI, 3 × 5 min; flush, 80%; purge, 300 s). Acetone was evaporated from the resulting extract at 60°C under nitrogen flushing and the residues were dried at 120°C overnight and weighed. The content of organic extractives was expressed per gram of original dry mass of wood.

The twice-extracted wood samples were further ground to a fine powder by a planetary ball mill (5 min, 650 rpm, Pulverisette7, Retsch, Idar-Oberstein, Germany). Subsequently, the content of Klason lignin was measured gravimetrically as dry weight of solids after sequential

hydrolysis with sulfuric acid [72% (v/v) at 30°C for 1 h and 2.4% (v/v) at 120°C for 1 h (Effland, 1977)]. Acid-soluble lignin was measured photometrically according to Dence (1992). The UV absorbance of the hydrolysate containing acid-soluble lignin was recorded at 205 nm ($\epsilon = 110 \text{ g}^{-1} \text{ cm}^{-1}$). Klason lignin and acid-soluble lignin contents were expressed per gram of original dry mass of wood. The water content was expressed as mass of water per unit of dry mass of wood. An overview of the wood parameters investigated is presented in Table 3.

Statistics

Microbial OTU richness was estimated from a normalized set of sequences (10 201) using rarefaction integrated in PAST (Hammer *et al.*, 2001). In six samples the amount sequenced was slightly below the threshold due to filtering of bad quality and unspecific sequences and an extrapolation was carried out using a power function in MS Excel ($R^2 > 0.99$).

All statistical analyses were performed using R 3.4.0 (R Core Team, 2017). For univariate statistical analyses, all response variables were tested for homogeneity of variance using the Levene's test in the R package 'car' (Fox and Weisberg, 2011). Additionally, the residuals of models were controlled visually for normality and homogeneity. In most cases, residuals seemed to be normally distributed by the fitted factors. Those variables that did not meet the assumptions of normality and homogeneity of variance were log transformed for analyses. In particular, the effect of tree species, wood compartment (i.e., sapwood vs. heartwood) and tree type (deciduous vs. coniferous wood) on microbial OTU richness was tested by three-way ANOVA. To investigate the effects of tree species and wood compartment on the main bacterial groups, two-way ANOVAs were undertaken separately for each phylum or proteobacterial class that accounted for $\geq 1.5\%$ of the entire data set. Detailed ANOVA results are given in Supporting Information Tables S5 and S6.

Multivariate statistical analyses of microbial community structure related to tree species, wood compartment and tree type were firstly assessed using permutational multivariate analysis of variance (three-way perMANOVA) (Anderson, 2001) based on Bray–Curtis distance using the function 'adonis' in the R package 'vegan' (Oksanen *et al.*, 2017). An advantage of perMANOVA is that it allows the inclusion of interactions between factors; however, it does not provide a graphical output. This analysis revealed significant difference between wood compartment (sapwood vs. heartwood), but no significant interaction of environmental factors. Hence, further multivariate analyses were performed separately for sapwood and heartwood as the main focus of this study. This was carried out by means of two-dimensional nonmetric

multidimensional scaling (NMDS) based on Bray–Curtis distance using the functions ‘metaMDS’ and ‘ordiellipse’ which add groups (tree species) onto the ordination plot. To assess correlations between community structure and wood parameters (pH, water content, Klason lignin, acid-soluble lignin, organic extractives and C/N ratio), variables were fitted as vectors onto the NMDS plots using the function ‘envfit’ with p values based on 999 permutations. To show the distribution of dominant OTUs, species scores were extracted from NMDS ordination and the 100 that were most abundant in the data set as a whole were plotted according to their respective positions in the ordination. Their averaged relative abundance within the sapwood and heartwood communities was also indicated by adjustment of circle sizes. Graphs were optically modified using CorelDRAW® Graphics Suite X8 (Corel Corporation, Ottawa, Canada).

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Author contributions

B.H., C.B., H.K., F.B. and M.H. conceived and designed the study; B.H., H.K. and S.L. performed field and laboratory work; B.H., E.S. and J.M. analysed the data; B.H., K and J.M. wrote the paper; A.D. supervised amplicon sequencing. All authors reviewed and edited the manuscript.

Data accessibility

All processed and merged OTU sequences have been submitted to the NCBI short read archive (SRA, [https://](https://www.ncbi.nlm.nih.gov/sra/)

www.ncbi.nlm.nih.gov/sra/) and are accessible under the number SRP102646.

Ethics approval and consent to participate

Field work permits were issued by the responsible environmental offices of the Free State of Thuringia (according to §72 BbgNatSchG).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1: Sequence counts of microbial groups for each wood sample.

Table S2: Species scores of the 100 most abundant OTUs for both NMDS axes and their averaged relative abundances in sapwood.

Table S3: Species scores of the 100 most abundant OTUs for both NMDS axes and their averaged relative abundances in heartwood.

Table S4: Primer sequence information applied in this study.

Table S5: Two-way ANOVA results for investigated wood parameters in relation to tree species and wood compartment (sapwood vs heartwood); Df - degrees of freedom; Sum Sq - sum of squares; Mean Sq - Mean of squares, significant factors are indicated in bold with $p < 0.05$.

Table S6: Two-way ANOVA results for dominant bacterial groups in relation to tree species and wood compartment (sapwood vs heartwood); Df - degrees of freedom; Sum Sq - sum of squares; Mean Sq - Mean of squares, significant factors are indicated in bold with $p < 0.05$.