Molecular monitoring of the poplar wood chip microbiome as a function of storage strategy

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ABSTRACT

One of the most challenging aspects of using wood chips as renewable energy source is the loss of biomass related to storage. Therefore, we installed three outdoor industrial-scale piles (250 m^3) of poplar wood chips and monitored the bacterial and fungal communities by next-generation sequencing over a storage period of 120 d. Two of the three piles were supplemented with calcium dihydroxide ($Ca(OH)_2$) (1.5%, 3% w/w) in order to test its potential as alkaline stabilization agent to preserve woody biomass during storage. Shifts in the microbial community composition occurred almost entirely in the beginning of the storage experiment, which we attribute to the temperature rise of up to 60 °C within the first week of storage. Later, however, we found little changes. Independent of $Ca(OH)_2$ concentration, a consortium of lignocellulolytic and thermotolerant microorganisms dominated the stored wood chip microbiota emphasizing their role as key players during wood decomposition. Although the addition of $Ca(OH)_2$ altered the physicochemical properties of wood chips, it did not prevent loss of biomass. Especially the pH was increased in $Ca(OH)_2$ treated piles. However, only minor differences in the microbial communities' composition were detected following $Ca(OH)_2$ addition, highlighting the microbes tolerance towards and adaptation to changing environmental conditions.

1. Introduction

On the basis of the Renewable Energy Directive 2018/2001/EU (RED II), the use of renewable energy is prospected to gradually increase, reaching a share of 32% of the total energy consumed in the European Union by the year 2030 (European Union, 2018). In comparison, in 2016, 17% of the gross final energy consumption were provided from renewables. Among these sources, energy derived from forestry is one of the major contributors, especially in the field of heating and cooling (European Environmental Agency, 2016; Scarlat et al., 2019). However, both forest operations and demand for woody biomass are seasonal and, therefore, some kind of storage is inevitable. After its shredding to chips, woody material is usually stored outdoors in large piles until it is consumed by a bioenergy facility (Wästerlund et al., 2017), for example a CHPP (Combined Heat and Power Plant). During wood chip storage, losses of both dry matter and fuel quality (calorific value) occur, representing the major problems of using biomass for energy supply (Noll and Jirjis, 2012). Recent studies reported dry matter losses (DML)

fluctuating between 6 and 27% during storage periods of six to twelve months in naturally ventilated outdoor piles (Afzal et al., 2010; Pecenka et al., 2014, 2018; Lenz et al., 2015; Whittaker et al., 2018). Reduction in dry matter is mainly caused by microbial degradation and physicochemical conversion processes, leading to heat release (Lenz et al., 2015). Due to the limited air passage inside the pile and the poor heat conductivity properties of the woody material, temperatures increase and in extreme cases self-ignition may occur (Jirjis, 2005; Ferrero et al., 2009, 2011; Noll and Jirjis, 2012). Interestingly, Ferrero et al. (2009) found that microbially-driven exothermic reactions are the main drivers of heat release, especially during the early stages of storage. Tree species (hardwood vs. softwood), process of wood shredding, particle size, composition of the woody material (heartwood, bark, needles), ambient weather conditions (temperature; solar radiation; precipitation), storage period, storage strategy (indoor; outdoor: covering vs. no covering; ventilation) and pile dimension represent factors altering wood chip quality and DML (Jirjis, 2005; Manzone et al., 2013; Barontini et al., 2014; Lenz et al., 2015; Pari et al., 2015; Pecenka et al., 2018; Eisenlauer

and Teipel, 2020).

Woody material is mainly composed of three types of polymers: cellulose, hemicellulose and lignin. Whereas cellulose and hemicellulose are macromolecules from different sugars, lignin originates from phenylpropanoid precursors forming aromatic polymers. These compositions do not only vary among different tree species, but also within a single plant, depending e.g. on its age or stage of growth (Pérez et al., 2002). The degradation of woody material is primarily conducted in an aerobic environment by a defined consortium of fungi and bacteria, possessing a variety of hydrolytic and oxidative enzymes (Pérez et al., 2002; Noll et al., 2010; Probst et al., 2018). Fungi, the primary wood decomposers, can be classified into white-, brown- and soft-rot fungi. White-rot fungi are characterized by their utilization of lignin and to a smaller extent hemicellulose and cellulose. In comparison, brown-rot fungi remove cellulose and leave lignin, albeit slightly modified. The third class of wood degrading fungi are soft-rot fungi, which need relatively high nitrogen levels for wood degradation and are able to grow under conditions too severe for brown or white-rot fungi (Daniel and Nilsson, 1998; Tuomela et al., 2000; de Boer et al., 2005). Even though bacteria are able to degrade woody compounds, their degradational activity seems to be negligible with respect to the fungal one, especially in aerobic environments (de Boer et al., 2005; Noll and Jirjis, 2012). However, recent evidence suggests synergies between bacteria and fungi in terms of N-supply during deadwood decomposition (Gómez-Brandón et al., 2017, 2020). Besides temperature, availability of nutrients (e.g. C:N ratio) and moisture content of the woody material, pH plays a pivotal role in microbial growth and degradational activity. Usually, fungi prefer acidic conditions, although a wide range of pH is tolerated by most species (Tuomela et al., 2000). Moreover, the majority of wood species are naturally acidic, showing pH values between 4.0 and 5.5 (Geffert et al., 2019). During wood degradation, microorganisms can cause a further acidification due to the production of organic acids (e.g. oxalic acid). Especially brown-rot fungi decrease wood pH, indicating degradational processes even before mass loss can be detected (Humar et al., 2001). With regards to minimizing microbial degradation of wood chips in storage piles, a more alkaline environment could inhibit microbial growth, a principle known as alkaline stabilization. Exemplarily, lime has been used for alkaline stabilization of biosolids for ages. More precisely, biosolids are treated with e.g. calcium dihydroxide ($Ca(OH)_2$) or calcium oxide (CaO) to raise the pH to 12 for at least 2 h (Bean et al., 2007; Williford et al., 2007).

In this study, we assessed and monitored the microbial community (bacteria and fungi) of poplar wood chip piles (*Populus canadensis*) at industrial scale (250 m^3) supplemented with different concentrations of $Ca(OH)_2$ during a storage period of 120 d by using Illumina MiSeq sequencing. Although the microbial community is mainly responsible for deadwood degradation, to our knowledge it has never been analyzed in detail in wood chip piles of industrial scale, especially not under different storage conditions. We aimed to i) determine the short- and long-term effects of $Ca(OH)_2$ addition on both the fungal and bacterial community and the related DML; and ii) characterize bacterial and fungal core communities as they are potential drivers of wood degradation. We hypothesized that the addition of $Ca(OH)_2$ changes physicochemical properties of the woody material, especially increases pH, to promote quality maintaining storage at industrial scale. Similar to the alkaline stabilization of biosolids, an elevated pH deteriorates environmental conditions mainly in terms of fungal growth as they prefer more acidic environments, leading to their inactivation. Consequently, the major key players of wood degradation are suppressed and we expected lower DML than in the wood chips stored without $Ca(OH)_2$.

2. Materials and methods

2.1. Experimental set-up

Ten years old poplar trees (*Populus canadensis*) grown as short

rotation coppice were harvested and stored at the BioEnergy Plant in Güssing, Austria (47°03′ N, 16°19′E) on April 1st and 2nd, 2019. Directly after chipping the woody material with a commercial mobile chipper (Musmax WD12, 100 × 100 mm), three piles of about 250 m^3 (12 m × 6 m × 3.5 m) were installed outdoors on an asphalted area (Fig. 1). Two of them were mixed with 1.5% and 3% $Ca(OH)_2$ (VERIT NATUR, Schretter & Cie GmbH & Co KG, Vils, Austria), respectively, whereas one pile was made up solely of wood chips without any amendment. The volume of each pile was defined by the number of full buckets of a wheel loader (8 m^3) and the respective amount of $Ca(OH)_2$ was based on the corresponding fresh mass of the wood chips, which was calculated by means of the bulk density (EN ISO 17828:2015). Subsequently, the blend was turned with the wheel loader for several times to guarantee a homogeneous mixture of the alkaline additive and poplar wood chips.

For continuous monitoring of principal storage conditions (e.g. temperature) and standardization of sampling, four stainless grit columns were positioned in each pile in intervals of 2 m (Fig. 1). The height, diameter and mesh size of the grit columns were 2.5 m, 0.64 m and 2 cm, respectively. In order to measure weight loss, 18 balance bags (plastic net bags, mesh size 1 × 1 mm) were filled with 2 kg of freshly harvested wood chips (mixed with the designed $Ca(OH)_2$ concentrations). After determining their exact weight, they were positioned at three different levels (0.8 m, 1.6 m, 2.4 m) within each column, resulting in six balance bags per height. Two measuring columns were equipped with a temperature data logger (Tinytag TGP-4017, Gemini Data Loggers, Chichester, United Kingdom) located at the height of 1.6 m. The third column contained two loggers at aforementioned height, whereas the fourth column was equipped with two loggers at each height level. Temperatures were monitored across all columns installed. For this study, environmental and molecular properties of samples taken after 35 (column 1) and 120 d of storage (column 4) were analyzed, respectively.

2.2. Sampling

In order to characterize the woody material at storage intake (t0), samples of poplar wood chips mixed with the designed concentrations of $Ca(OH)_2$ were taken immediately after assembling the piles: 20 representative samples of each pile were randomly collected to determine the moisture content/dry matter and three samples were collected for physicochemical and molecular analysis. To assess storage effects, samples were taken after 35 d of outdoor storage without any coverage and at the end of the large-scale experiment, after 120 d, representative for short- and long-term storage, respectively. For sampling, one measuring column of each pile (0%, 1.5%, 3% $Ca(OH)_2$) was removed with a truck crane and the resulting free spaces were filled with wood chips. The balance bags were removed, weighted and aliquots of approximately 300 g were taken to determine moisture content/dry matter loss (DML). For physicochemical and molecular analysis, aliquots (approx. 500 g) of two balance bags were pooled equally. The resulting three samples per height were stored at 4 °C and transported to the laboratory, where they were crushed and pulverized by using a cutting-mill (2 mm, Pulverisette 19, Fritsch, Idar-Oberstein, Germany). Aliquots of each sample were stored at −20 °C and −80 °C for physicochemical and molecular analysis, respectively.

2.3. Physicochemical properties

Cut-milled samples were oven-dried at 105 °C for 20 h to determine their moisture content. The content of volatile solids (VS) was calculated from the weight loss of oven-dried samples after ignition at 550 °C for 5 h in a muffle furnace (Carbolite CWF 1000, Carbolite Gero, Germany). Additionally, oven-dried material was used to analyze total C and N contents using a CN analyzer (TruSpec, Leco, Michigan, USA). Electrical conductivity (EC) and pH of cut-milled samples were determined in wood:water extracts (1:20, w/v) incubated at room temperature
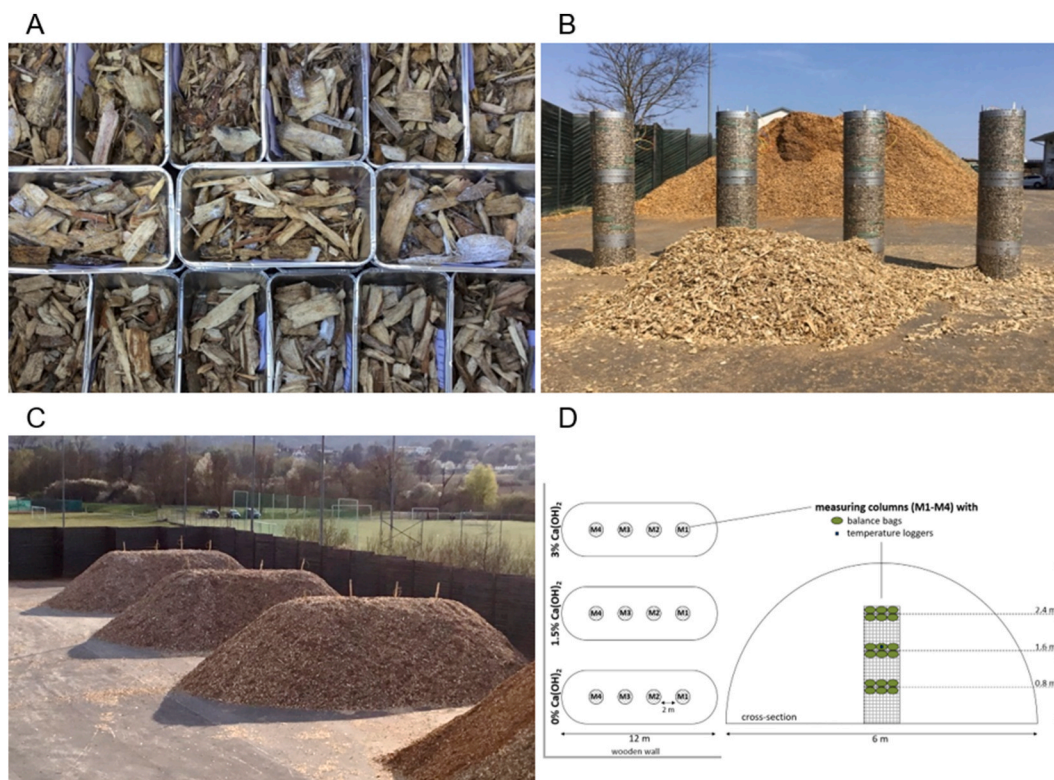
Fig. 1. Experimental set-up of the large-scale trial. (A) Freshly harvested poplar wood chips. (B, C) Assemblage of the wood chip piles (250 m³) containing measuring columns. (D) Schematic structure of the storage piles without alkaline amendment (0%) and supplemented with 1.5% and 3% Ca(OH)₂. Columns 1 and 2 were equipped with one temperature data logger (D), whereas column 3 contained two loggers at the height of 1.6 m. Column 4 was equipped with two loggers at each height level. Columns 1 and 4 were removed after 35 and 120 d of storage, respectively. In the present study, samples from columns 2 and 3 were only considered for the temperature monitoring.

overnight using a conductivity meter (LF 330 WTW, Weilheim, Germany) and a pH meter (Metrohm 826 pH mobile, Metrohm, Switzerland), respectively.

2.4. Dry matter loss

DML was calculated according to Lenz et al. (2015) considering moisture content of the woody material at storage intake and outtake. Due to the addition of the alkaline additive, the corresponding formula was slightly modified: In a wet environment, Ca(OH)₂ reacts with carbon dioxide (CO_2), forming calcium carbonate ($CaCO_3$), water and heat (Moorehead, 1986). As a result, the mass of the applied additive increases. Assuming an entire conversion of Ca(OH)₂ to $CaCO_3$ during the storage of wood chips, all masses at storage outtake were corrected for the mass of $CaCO_3$ instead of Ca(OH)₂ (Equation 1). However, measuring inaccuracies and deviations in the calculations can result in high standard deviations or even negative values of calculated DML.

$$DML = (1-(m_{out}(100-x_{out})-m_{Ca(OH)2})/(m_{in}(100-x_{in})-m_{CaCO3}))*100$$

DML dry matter loss [%]
m_{in} wet mass at storage intake [kg]
m_{out} wet mass at storage outtake [kg]
x_{in} moisture content at storage intake [%]
x_{out} moisture content at storage outtake [%]
$m_{Ca(OH)2}$ dry mass of added Ca(OH)₂ at storage intake [kg]
m_{CaCO3} dry mass of formed $CaCO_3$ at storage outtake [kg]

2.5. Molecular analysis

2.5.1. DNA extraction and Illumina MiSeq sequencing

Extraction and purification of whole community DNA was performed with the NucleoSpin®Soil kit (Macherey-Nagel) according to the manufacturer's protocol with some minor modifications: one ceramic sphere (6.35 mm (¼"), MP Biomedicals) was added to the lysing tubes to achieve an accurate disruption of the woody tissue using a FastPrep®-24 Instrument (MP Biomedicals) together with Buffer SL2. To increase the overall DNA yield, two technical replicates of each sample (à 0.1 g) were lysed separately and pooled on the NucleoSpin®Soil Column, resulting in one eluate per sample. The concentration of extracted double-stranded DNA was quantified fluorometrically (Quantus Fluorometer, Promega). Microbial communities were assessed using an Illumina MiSeq 2 × 250 bp paired-end sequencing approach (Microsynth AG, Switzerland). For bacteria, the V4 region of the 16S rRNA gene was sequenced using the primer pair 515F/806R (Caporaso et al., 2011). For fungi, the ITS2 region was sequenced using the primer pair ITS3/ITS4 (White et al., 1990). Raw sequencing data (primers and adapters removed) were deposited in Sequence Read Archive (SRA) under the submission number PRJNA669574.

2.5.2. Next generation sequencing data analysis

Trimmed Illumina reads (.fastq) were analyzed in R v.3.6.2 (R Core Team, 2019) using the package dada2 v.1.14.1 (Callahan et al., 2016). Briefly, bacterial and fungal reads were filtered allowing a maximum of two expected errors per read (maxEE = 2). Two samples showed a low quality for both the V4 and ITS2 region and therefore, they were excluded from further analysis. Following error rate learning and inference of amplicon sequence variants (ASVs), forward and reverse sequences were merged and a sequence frequency table was generated.

Chimeras were removed and unique V4 and ITS2 sequences were aligned to the SILVA v.132 (Callahan, 2018) and UNITE v.8.0 (UNITE Community, 2019) database, respectively. V4 sequences assigned to either the kingdom Eukaryota, the order Chloroplast or the family Mitochondria were removed. In addition, sequences unassigned at kingdom level were discarded. Hence, the analysis resulted in two ASV tables listing the total read abundances of all detected fungal and bacterial ASVs for each sample. The final tables contained 2,904 bacterial and 756 fungal ASVs. In both cases, a typical long-tailed distribution with a high number of low abundant/rare ASVs was observed. For bacteria, the sequencing depth over all samples varied between a minimum of 21,704 and a maximum of 115,141 paired reads. Comparing the sequencing depth of sample groups, no significant differences were found as tested by Kruskal-Wallis (p_{time} = 0.622, $p_{Ca(OH)_2}$ = 0.070, $p_{column\ level}$ = 0.092). For fungi, minimum and maximum sequencing depth were 2,348 and 43,203, respectively. There were no differences in the sequencing depth between the $Ca(OH)_2$ concentrations (p = 0.133) and the sampling heights (p = 0.900), however, the sequencing depth differed between the time points (p = 0.006). According to post-hoc Kruskal-Dunn test, significant differences occurred solely between samples collected after 35 d (24,545 ± 9,680 reads) and 120 d (16,165 ± 8,869 reads).

2.6. Statistical analysis

In order to test the effect of $Ca(OH)_2$ addition (0%, 1.5%, 3%), storage time (t0, 35 d, 120 d) and sampling height within each measuring column (0.8 m, 1.6 m, 2.4 m) on environmental variables, non-parametric Kruskal-Wallis tests were executed in R v.3.6.2 (R Core Team, 2019) and pairwise comparisons were performed using post-hoc tests according to Dunn using the package PMCMR v.4.3 (Pohlert, 2014). Significance was determined at p ≤ 5%. The richness of bacterial and fungal ASVs was estimated using the package breakaway v.4.6.16 (Willis et al., 2019). Breakaway uses the distribution of observations across individual samples composing a sample group in order to estimate the number of observations in a sample group. As this idea is conceptual, the unit of observations can be species, ASVs, OTUs or the like. The resulting richness estimate reflects the input unit. Due to intraspecies variation, a biological species might be represented by more than a single ASV (or OTU) and the estimated richness might not accurately reflect the total number of species. Beta diversity analysis have been shown to be robust independent of the species-concept applied (Glassman and Martiny, 2018). Non-metric multidimensional scaling of microbial communities was conducted with the package vegan v.2.5–6 (Oksanen et al., 2019) based on Bray-Curtis dissimilarity. The influence of categorical factors and continuous environmental properties on the dissimilarity matrix was modelled using permutational multivariate analysis of variance (Adonis). To estimate the effect of storage time on the bacterial and fungal compositions, a generalized linear model of centered log-ratio (clr) transformed count data was calculated using the package ALDEx2 v.1.18.0 (Gloor et al., 2016). In the same manner, effects of $Ca(OH)_2$ concentration on the microbial community of long-term stored (120 d) wood chips were analyzed. Environmental variables with significant impact on the dissimilarity matrix were correlated (Spearman) with all ASVs using the aldex.corr function. ASVs with significant Benjamini-Hochberg corrected p-values (≤5%) were interpreted. Venn diagrams were computed with the package VennDiagram v.1.6.20 (Chen, 2018). Prior to Venn analysis, data were filtered to remove ASVs which were not representative for the sample group: For each time point (t0, 35 d, 120 d) and $Ca(OH)_2$ concentration (0%, 1.5%, 3%), those ASVs were removed which were not detected in at least 3 out of 9 samples. At storage intake, different concentrations of $Ca(OH)_2$ were not considered as samples were taken instantly after mixing the biomass-additive blends and no immediate effect on the analysis of the microbial community was expected. In addition, ASVs with less than 5 reads within these sample groups were removed prior to analysis.

Procrustes analysis showed that filtering did neither affect bacterial (m^2 = 0.085, p = 0.001) nor fungal community (m^2 = 0.026, p = 0.001). Figures were produced in R using the packages phyloseq v.1.30.0 (McMurdie and Holmes, 2013), dplyr v.0.8.5 (Wickham et al., 2020), ggplot2 v.3.3.0 (Wickham, 2016) and extrafont v.0.17 (Chang, 2014). Multiple plots were arranged partially using the packages ggpubr v.0.3.0 (Kassambara, 2020) and gridExtra v.2.3 (Auguie, 2017).

3. Results

The effect of $Ca(OH)_2$ amendment as alkaline stabilizer on short- (35 d) and long-term (120 d) stored poplar wood chips was evaluated at industrial scale. Due to the large scale (250 m^3 storage piles), heterogeneity within each pile was expected. In order to assess this heterogeneity, samples were taken at three different levels within each measuring column. For the assessed environmental variables (pH, EC, moisture, total C, total N, C:N, VS, DML), the effect of sampling height was negligible (p > 0.05). We concluded that the effect of sampling height did not mask potential effects of storage time and $Ca(OH)_2$ addition. Therefore, pile heterogeneity will not be discussed in the following sections. In rare cases, in which the height had a relevant impact (e.g. temperature), pile heterogeneity is addressed explicitly.

3.1. Environmental variables

At storage intake (t0), the addition of $Ca(OH)_2$ immediately changed the physicochemical properties of the poplar wood chips: pH (p = 0.027) and EC (p = 0.027) were higher in both biomass-additive blends (1.5% and 3%) than in the woody material without $Ca(OH)_2$, whereas VS content was decreased (p = 0.039). For the other variables, no immediate effects of $Ca(OH)_2$ were observed (Table 1). Independent of $Ca(OH)_2$ concentration, all physicochemical properties changed over time, except VS and total C content. While the pH (p = 0.003) and C:N ratio (p < 0.001) increased, EC (p < 0.001), moisture (p < 0.001) and total N content (p < 0.001) decreased. Due to the buffering capacity of the alkaline additive, the increase in pH was strongest in the pile without $Ca(OH)_2$ addition, reaching its highest pH of 6.7 at the end of the large-scale experiment. Likewise, the highest decrease in moisture content was observed without any amendment (Table 1). In contrast, EC and total N content showed strongest decrease in the pile supplemented with 3% $Ca(OH)_2$. In order to evaluate the long-term effect of $Ca(OH)_2$ on poplar wood chips, physicochemical properties of samples taken after 120 d of storage were compared. Significant differences were detected for pH (p < 0.001), EC (p < 0.001), moisture (p = 0.032), total C (p = 0.019) and VS content (p < 0.001). Indeed, performing paired comparisons, pH and VS were the only variables showing concentration dependent differences between all three piles.

The temperatures within the piles gradually started to increase immediately after the assemblage of the piles reaching a maximum of 61.9 °C, 62.6 °C and 66.9 °C in the piles without (0%) and supplemented with 1.5% and 3% $Ca(OH)_2$, respectively (Fig. 2). Without the alkaline amendment, the maximum temperature was reached on average 6 d after storage intake, whereas in presence of $Ca(OH)_2$ maximum temperatures were reached two days earlier. After reaching maximum temperature, a cooling phase followed by a second temperature rise was observed for all piles. Finally, the temperature stabilized at approximately 30 °C after 60 d of storage independent of $Ca(OH)_2$ addition. Highest temperatures were reached in the upper parts, while they were lowest close to the ground. As the outer surface of the piles was more directly exposed to the surrounding (weather) conditions than the inner parts, the temperature gradient was an inherent characteristic of this large-scale approach. Nevertheless, pile temperature did not cool to ambient temperature (Fig. 2).

The addition of $Ca(OH)_2$ increased dry matter loss (DML) of short-term stored wood chips (35 d) (p < 0.001). For the piles supplemented with 1.5% and 3% $Ca(OH)_2$ mean DML of 8.3% (± 3.12) and

Table 1

Physicochemical properties of poplar wood chips collected at storage intake (t0), after 35 and 120 d of storage from the piles without alkaline amendment (0%; P0) and supplemented with 1.5% (P1.5) and 3% (P3) Ca(OH)$_2$. Values are means with the standard deviations (in brackets).

|		pH	EC [mS cm^{-1}]	moisture [%]	total C [%]	total N [%]	C:N	VS [%]
t0	P0	5.2 (0.27)	0.27 (0.042)	48 (3.0)	47 (0.3)	0.21 (0.151)	470 (529.2)	98 (0.3)
	P1.5	6.5 (0.16)	0.69 (0.021)	47 (3.7)	46 (1.5)	0.32 (0.127)	170 (93.5)	96 (0.2)
	P3	7.2 (0.45)	0.85 (0.070)	47 (3.1)	45 (0.6)	0.41 (0.046)	111 (11.8)	95 (1.0)
35 d	P0	6.0 (0.10)	0.20 (0.024)	27 (3.3)	47 (0.8)	0.28 (0.058)	173 (37.3)	98 (0.4)
	P1.5	7.4 (0.11)	0.31 (0.035)	38 (4.1)	46 (0.6)	0.33 (0.064)	145 (31.2)	97 (0.5)
	P3	7.7 (0.07)	0.31 (0.026)	37 (3.8)	45 (0.5)	0.26 (0.074)	185 (47.0)	96 (0.5)
120 d	P0	6.7 (0.18)	0.18 (0.021)	27 (5.0)	47 (0.6)	0.15 (0.109)	2079 (4433.0)	98 (0.3)
	P1.5	7.6 (0.11)	0.26 (0.020)	33 (3.1)	46 (0.4)	0.18 (0.073)	285 (102.5)	97 (0.4)
	P3	8.0 (0.20)	0.26 (0.031)	32 (3.3)	46 (0.7)	0.19 (0.053)	259 (73.6)	96 (0.9)
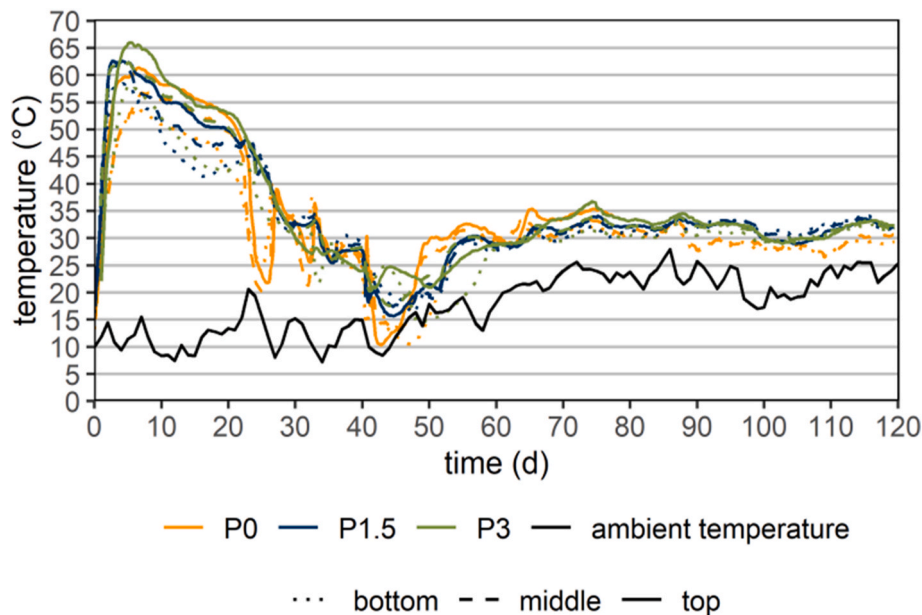
Fig. 2. Temperature in poplar wood chip piles (250 m^3) without alkaline amendment (0%; P0), and supplemented with 1.5% (P1.5) and 3% (P3) Ca(OH)$_2$ during a storage period of 120 d. Temperatures were measured at three different heights within each pile: 0.8 m (bottom), 1.6 m (middle), 2.4 m (top). Ambient temperature was indicated according to ZAMG (Central Institute for Meteorology and Geodynamics).

2.7% ($\pm$5.44) were calculated, respectively, while there was no decrease in the unamended pile. Contrarily, after long-term storage of poplar wood chips (120 d), DML was comparable for all piles (p = 0.546), reaching mean values of 6.9% ($\pm$4.53), 8.9% ($\pm$3.19) and 8.6% ($\pm$2.89) in the presence of 0%, 1.5% and 3% Ca(OH)$_2$, respectively.

3.2. Bacterial composition

The bacterial community was generally dominated by Actinobacteria (36% of all reads, 548 ASVs), followed by Proteobacteria (34%, 1,084 ASVs), Firmicutes (21%, 411 ASVs) and Bacteroidetes (7.5%, 404 ASVs).

Both the alkaline additive and storage time significantly affected the bacterial community. Comparing the bacterial compositions of all samples, 41% of the explained variance was attributed to storage time (p$_{Adonis}$ = 0.001), whereas the effect of Ca(OH)$_2$ was half as large (19% of variance, p$_{Adonis}$ = 0.001) (Figure S1). Independent of Ca(OH)$_2$ concentration, the number of ASVs was highest after long-term storage. However, estimated ASV richness was 4,226 (lower bound = 1,678; upper bound = 261,781), 1,478 (965; 11,845) and 4,970 (1,217; 523,023) at storage intake, after 35 d and 120 d, respectively. Regardless of Ca(OH)$_2$ concentration, just a small number of 25 out of 2,904 ASVs (0.9%) was found in common at all time points, emphasizing the dynamic shift of the bacterial community over time (Fig. 3A and B). This bacterial core community was mainly composed of species affiliated to

the orders Enterobacteriales, Pseudomonadales and Streptomycetales. However, a generalized linear model based on storage time without considering Ca(OH)$_2$ concentration revealed significantly different relative abundances between freshly harvested and stored wood chips (Table S1). At storage intake, the bacterial community was dominated by Enterobacteriales, e.g. *Yersinia*, *Klebsiella*, *Pantoea* and unassigned Enterobacteriaceae, as well as members of the order Pseudomonadales, mainly *Pseudomonas*, showing mean relative abundances of 40% and 34%, respectively. After short- (35 d) and long-term storage (120 d) of poplar wood chips, their abundance was dramatically decreased and together they accounted for < 3% and 0.5% of all reads, respectively. In contrast, Streptomycetales, e.g. *Streptomyces*, reached their highest mean relative abundance after 120 d (12%), whereas at storage intake 0.06% of all reads were affiliated to this taxon. Supporting the thermal effect observed before, the effect of storage duration appeared to be minor (Fig. 3A, Table S1). Time-dependent differences in ASV abundances were usually observed between storage intake and stored material: Out of the 194 ASVs (6.7% of the total ASVs), whose abundances differed significantly between the time points, 169 ASVs (5.8% of the total ASVs) were significantly lower/higher in abundance at storage intake compared to the wood chips stored for 35 d and/or 120 d while their abundances were comparable between short- and long-term storage. Moreover, 89% out of 442 ASVs observed at storage intake were not detected anymore after 35 d. Regardless of the bacterial core community found at all time points, 78% of the ASVs detected after 35 d were found
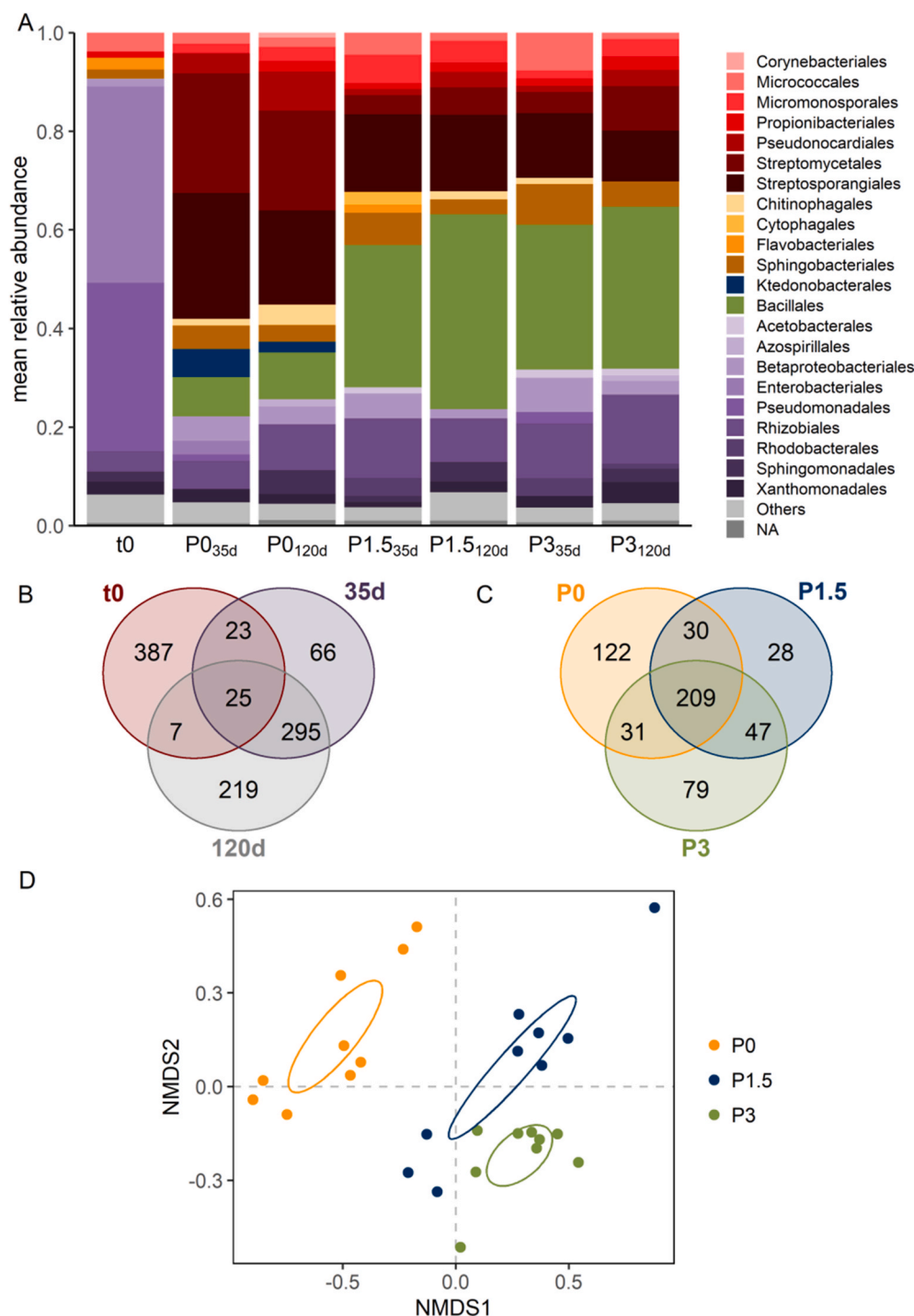
Fig. 3. Bacterial composition of poplar wood chips. (A) Mean relative abundances of bacterial ASVs obtained by Illumina MiSeq profiling at order level in poplar wood chip samples taken at storage intake (t0), and after 35 and 120 d of storing the piles without alkaline amendment (0%; P0) and supplemented with 1.5% (P1.5) and 3% (P3) $Ca(OH)_2$. Orders representing Actinobacteria, Bacteroidetes and Proteobacteria are shown in red, orange and violet shades, respectively. Ktedonobacterales representing Chloroflexi and Bacillales representing Firmicutes are depicted in blue and green, respectively. Bacterial orders <1% are shown as "Others" and those which remained unclassified at order level are classified as "NA". (B, C) Venn diagrams illustrating unique and common bacterial ASVs according to storage duration (B), and $Ca(OH)_2$ concentration after a storage time of 120 d (C). (D) Non-metric multidimensional scaling (NMDS) of the bacterial community data obtained by Illumina MiSeq profiling after 120 d of poplar wood chip storage based on Bray Curtis similarity (stress = 0.111). Ellipses embracing sample groups were drawn at a confidence level of 95% around group centroid for samples taken from the piles without alkaline amendment (0%; P0) and supplemented with 1.5% (P1.5) and 3% (P3) $Ca(OH)_2$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

after 120 d as well (Fig. 3B), showing mean relative abundances of 96% and 90%, respectively. This supports the observation that the duration of storage time did not have a major impact on the microbiota of the piles. Thereof, the most abundant species were classified as *Streptomyces* (mean relative abundance$_{35d,120d}$ = 4–24%), *Actinomadura* (5–20%), *Nonomuraea* (1–5%) and unclassified Micromonosporaceae (2–6%, Actinobacteria), *Brevibacillus* (2–14%), *Laceyella* (0.6–6%) and *Thermoactinomyces* (0.4–8%, Firmicutes), *Ochrobactrum* (1–7%) and unclassified Rhizobiaceae (0.8–7%, Proteobacteria) and *Olivibacter* (2–7%, Bacteroidetes). Several bacterial families were detected exclusively in stored material without finding any of their members in freshly harvested wood chips, e.g. Thermomonosporaceae (Actinobacteria) or

Thermoactinomycetaceae (Firmicutes).

In order to evaluate the effect of $Ca(OH)_2$ addition on the bacterial community, samples from the initial time point were excluded from the analysis and only samples taken after short- (35 d) and long-term (120 d) storage were considered. In agreement with the high similarity in the bacterial composition observed between short- and long-term storage, the effect size of $Ca(OH)_2$ concentration (34% of variance, p_{Adonis} = 0.001) was almost three times higher than the effect size of storage time (13%, p_{Adonis} = 0.001) (Figure S2). Over time, the effect of the alkaline additive gained importance and after 120 d, 43% of the explained variance were attributed to $Ca(OH)_2$ concentration (p_{Adonis} = 0.001) (Fig. 3D). Effect sizes of physicochemical variables on the bacterial

composition were calculated in order to link the bacterial community to environmental properties. Underlining the effect of Ca(OH)$_2$, out of the seven variables tested (pH, EC, moisture, total C, total N, C:N, VS), only pH (32%, p_{Adonis} = 0.001) and moisture (12%, p_{Adonis} = 0.002) explained more than 10% of the variance.

To further pinpoint differences in the bacterial composition primarily caused by the alkaline additive, solely samples taken after long-term storage were considered, thereby removing the effect of storage time. Hence, 546 ASVs were detected after long-term storage of poplar wood chips, of which 38% were found irrespective of Ca(OH)$_2$ concentration, accounting on average for 81%, 88% and 86% of all reads in the piles supplemented with 0%, 1.5% and 3% Ca(OH)$_2$ (Fig. 3C). Interestingly, these ASVs were affiliated to the same bacterial families as the above-described bacterial community of short- and long-term stored wood chips, e.g. Streptomycetaceae, Thermomonosporaceae, Pseudonocardiaceae, Streptosporangiaceae and Micromonosporaceae (Actinobacteria), Paenibacillaceae and Thermoactinomycetaceae (Firmicutes), Rhizobiaceae and Sphingomonadaceae (Proteobacteria) and Sphingobacteriaceae (Bacteroidetes). At finer taxonomic scale, *Streptomyces* (mean relative abundance$_{120d}$ = 5–17%), *Actinomadura* (5–12%), *Nonomuraea* (1–5%) and unclassified Micromonosporaceae (2–4%, Actinobacteria), *Brevibacillus* (4–14%), *Laceyella* (0.6–6%) and *Thermoactinomyces* (0.2–5%, Firmicutes), *Bordetella* (1–2%) and unclassified Rhizobiaceae (4–6%, Proteobacteria) and *Olivibacter* (2–4%, Bacteroidetes) were identified as the most abundant taxa. Contrarily, the abundances of ASVs occurring exclusively in one of the three piles were quite low, showing mean relative abundances of 8%, 1% and 2% in the piles with 0%, 1.5% and 3% Ca(OH)$_2$, respectively.

Modelling the effect of Ca(OH)$_2$ concentration on the bacterial community using a generalized linear model, 13 ASVs (0.5% of the total ASVs) had significant higher/lower abundances in presence of the alkaline additive compared to the wood chips stored without Ca(OH)$_2$ (Table S2). They were unaffected by its concentration, showing comparable abundances in the piles supplemented with 1.5% and 3% Ca(OH)$_2$. Exemplarily, species annotated as *Laceyella* and *Thermoactinomyces* (Firmicutes) were most abundant in the presence of the alkaline additive, whereas ASVs affiliated to e.g. *Streptomyces* and *Kribbella* (Actinobacteria), *Chitinophaga* and *Filimonas* (Bacteroidetes) or *Thermosporothrix* (Chloroflexi) seemed to thrive without Ca(OH)$_2$, of which 5 ASVs exclusively occurred in that pile. Three ASVs annotated as *Brevibacillus*, unclassified Bacillaceae (Firmicutes) and unclassified JG30-KF-CM45 (Chloroflexi), respectively, were found to significantly differ between samples taken from the unamended pile (0%) and the pile supplemented with 1.5%, but not between 0% and 3% Ca(OH)$_2$. Moreover, 17 ASVs were only promoted/inhibited by the highest concentration of the alkaline additive (3%), observing no significant differences between 0% and 1.5%. They were classified as members of e.g. *Streptomyces* and *Jiangella* (Actinobacteria), *Thermoactinomyces* and *Bacillus* (Firmicutes), unclassified Rhizobiaceae, *Devosia*, *Chthonobacter* and *Chelativorans* (Proteobacteria), *Parapedobacter* (Bacteroidetes) and *Thermosporothrix* (Chloroflexi). Apart from the latter one, all these ASVs showed their highest relative abundance at the highest Ca(OH)$_2$ concentration. Furthermore, 6 out of these 17 ASVs (*Streptomyces*, unclassified Rhizobiaceae, *Devosia*, *Chthonobacter*, *Parapedobacter*) were found to significantly differ not only between 0% and 3% Ca(OH)$_2$, but also between 1.5% and 3% Ca(OH)$_2$, clearly showing a concentration dependent increase in relative abundances with increasing Ca(OH)$_2$ addition. Overall, out of these 33 ASVs, which were significantly influenced by Ca(OH)$_2$ concentration as found by the generalized linear model, 32 either positively or negatively correlated with pH, highlighting the main effect of Ca(OH)$_2$ on this variable (Table S3).

3.3. Fungal composition

Out of the 756 fungal ASVs detected, 538 were classified as members of the phylum Ascomycota, accounting for almost 97% of all sequences.

Despite their low abundances in comparison to the Ascomycota, 130 Basidiomycota (3% of all reads) and 29 Mucoromycota ASVs (0.15% of all reads) were found.

Shifts in the fungal composition of all samples were mainly caused by the effect of storage time, whereas Ca(OH)$_2$ concentration played a minor role. In detail, the time of storage explained 34% of the total variance (p_{Adonis} = 0.001), while the effect of Ca(OH)$_2$ concentration was negligible (7%, p_{Adonis} = 0.001), emphasizing a shift in the fungal community over time (Figure S3). The number of detected ASVs was highest at storage intake. Independent of Ca(OH)$_2$ addition, estimated ASV richness was 1,542 (lower bound = 644; upper bound = 91,986), 291 (74; 27,771) and 498 (125; 50,733) at storage intake (t0) and after short- (35 d) and long-term storage (120 d), respectively. *Aspergillus fumigatus* (Eurotiomycetes) and *Dipodascus geotrichum* (Saccharomycetes) were the only two ASVs which were found not only at storage intake but also in stored material, indicating an almost entire change of the fungal community over time (Fig. 4A and B). The latter one was equally abundant at all time points, showing mean relative abundances of less than 0.02%. On the other hand, the mean relative abundance of *A. fumigatus* was highest in short-term stored material (12%), whereas at storage intake the mean relative abundance (0.05%) was comparably low as supported by the results of the generalized linear model (Table S4).

At storage intake, the fungal community was mainly composed by a consortium of 174 ASVs exclusively found at this sampling point (Fig. 4B), reaching a mean relative abundance of 87%. In detail, the most abundant genera were classified as members of the phyla Basidiomycota and Ascomycota, e.g. *Vishniacozyma* (mean relative abundance$_{t0}$ = 10%) and *Hannaella* (4%, Tremellomycetes, Basidiomycota), *Cladosporium* (12%), *Botryosphaeria* (4%), *Pyrenochaeta* (3%, Dothideomycetes), *Knufia* (5%, Eurotiomycetes), *Lecania* (4%, Lecanoromycetes) and *Candida* (4%, Saccharomycetes, Ascomycota). In line with the high ASV richness at storage intake, numerous fungal orders were detected solely in this sample group, e.g. Tremellales (Tremellomycetes), Capnodiales, Botryosphaeriales and Dothideales (Dothideomycetes), Phaeomoniellales (Eurotiomycetes), Lecanorales and Caliciales (Lecanoromycetes) and Xylariales (Sordariomycetes). Contrarily, after 35 d of storage, the fungal community was dominated by a small number of 5 ASVs belonging to the phylum Ascomycota. On average, they accounted for 99% of all reads. Besides *A. fumigatus* (mean relative abundance$_{35d}$ = 8–20%), which had already been found at storage intake, the remaining 4 ASVs emerged during the storage of poplar wood chips. They were affiliated to two different families, Trichocomaceae (Eurotiomycetes), more specifically *Thermomyces lanuginosus* (mean relative abundance$_{35d}$ = 35–70%) and *Rasamsonia emersonii* (1–7%), and Chaetomiaceae (19–37%, Sordariomycetes). Together, these 5 ASVs showed a mean relative abundance of 89% after long-term storage, suggesting the formation of a stable community independent of storage duration.

To assess the effect of Ca(OH)$_2$ concentration on the fungal community, the dataset was reduced to samples taken after 35 and 120 d, thereby disregarding the major shift between freshly harvested and stored wood chips. As expected, the effect size of storage time was almost completely reduced (7% of variance, p_{Adonis} = 0.003), while there was a small, yet significant effect of Ca(OH)$_2$ addition (11%, p_{Adonis} = 0.001) (Figure S4). At the end of the large-scale experiment (120 d), however, the effect size of Ca(OH)$_2$ addition was similarly low, explaining 13% of the variance (p_{Adonis} = 0.049) (Fig. 4D). Moreover, the linkage between environmental properties and the fungal composition confirmed these results: each of the seven variables tested (pH, EC, moisture, total C, total N, C:N, VS) explained < 10% of the variance in the samples taken after 120 d of storage.

As for the bacterial community, solely samples taken after long-term storage were considered to evaluate differences caused by Ca(OH)$_2$ concentration. Hence, 31 ASVs were detected, of which 7 occurred in all three piles (i.e. at all concentrations), forming a fungal core community, which was unaffected by Ca(OH)$_2$ concentration (Fig. 4C). On average,
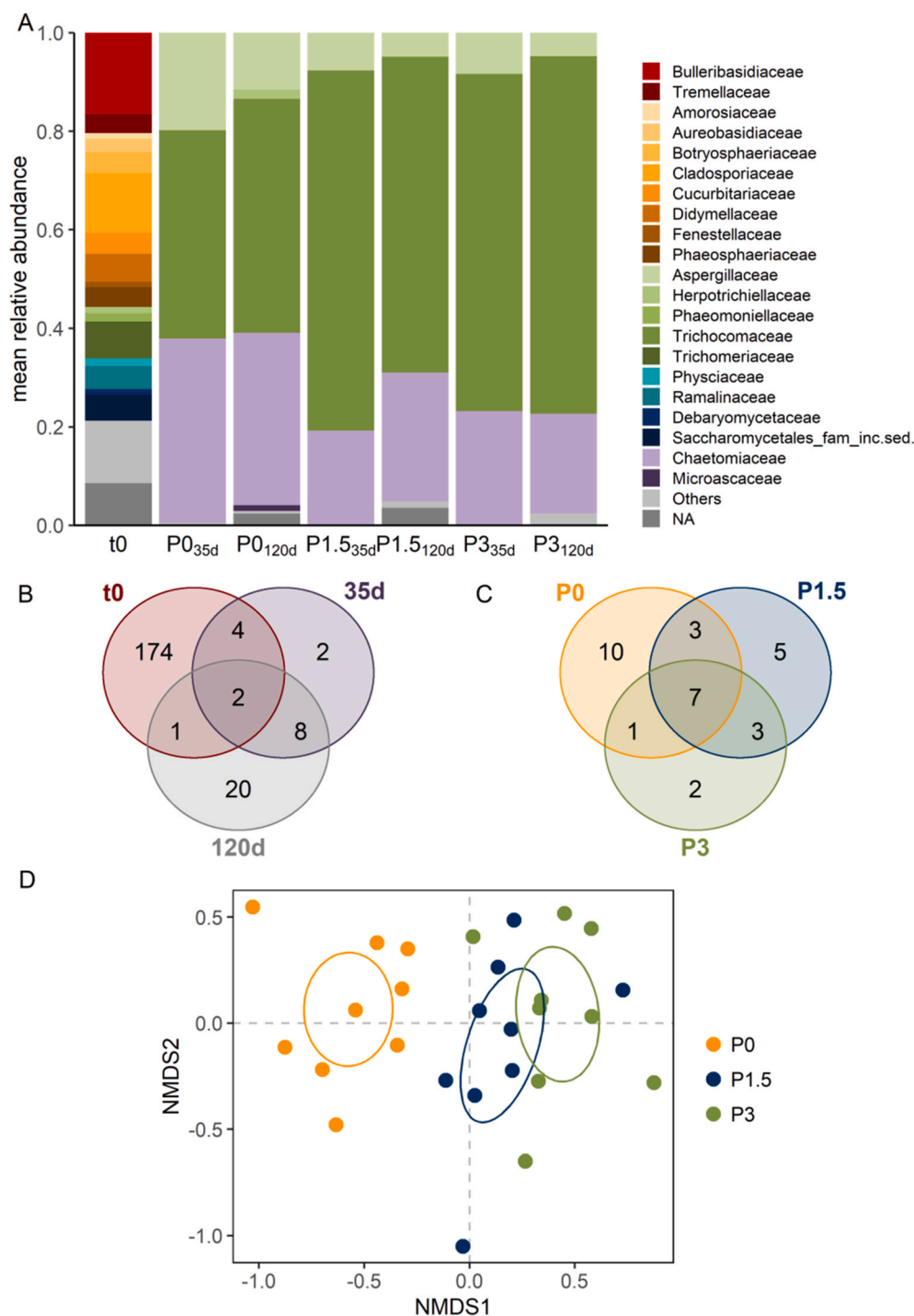
Fig. 4. Fungal composition of poplar wood chips. (A) Mean relative abundances of fungal ASVs obtained by Illumina MiSeq profiling at family level in poplar wood chip samples taken at storage intake (t0), and after 35 and 120 d of storing the piles without alkaline amendment (0%; P0) and supplemented with 1.5% (P1.5) and 3% (P3) $Ca(OH)_2$. Families representing Tremellomycetes (Basidiomycota), Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Saccharomycetes and Sordariomycetes (Ascomycota) are shown in red, orange, green, turquoise, blue and violet shades, respectively. Fungal families <1% are shown as "Others" and those which remained unclassified at family level are classified as "NA". (B, C) Venn diagrams illustrating unique and common fungal ASVs according to storage duration (B), and $Ca(OH)_2$ concentration after a storage time of 120 d (C). (D) Non-metric multidimensional scaling (NMDS) of the fungal community data obtained by Illumina MiSeq profiling after 120 d of poplar wood chip storage based on Bray Curtis similarity (stress = 0.208). Ellipses embracing sample groups were drawn at a confidence level of 95% around group centroid for samples taken from the piles without alkaline amendment (0%; P0) and supplemented with 1.5% (P1.5) and 3% (P3) $Ca(OH)_2$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

these fungal core ASVs accounted for 70%, 88% and 95% of all reads in the unamended pile (0%) and those supplemented with 1.5% and 3% Ca (OH)$_2$, respectively. Thereof, the 5 most abundant ASVs were affiliated to the same fungal families already dominating the core community of stored wood chips, namely Trichocomaceae, Aspergillaceae and Chaetomiaceae (Fig. 4A). At finer taxonomic scale, they were affiliated to *T. lanuginosus* (mean relative abundance$_{120d}$ = 39–64%), *R. emersonii* (3–7%), *R. argillaceae* (0.2–2%), *A. fumigatus* (4–11%, Eurotiomycetes) and unclassified Chaetomiaceae (14–20%). For the sake of completeness, the remaining two ASVs were annotated as *Capronia semi-immersa* (Eurotiomycetes) and unclassified Orbiliaceae (Orbiliomycetes). The mean relative abundance of unique ASVs was highest in the pile without

any amendment (6%) and therefore, they are suggested to be influenced by the alkaline additive. However, the corresponding generalized linear model based on $Ca(OH)_2$ concentration revealed no ASVs which showed a significantly higher or lower abundance due to the addition of the alkaline additive. Hence, in comparison to the bacterial community, the fungal community appeared unaffected by the addition of $Ca(OH)_2$.

4. Discussion

The amendment of the alkaline additive $Ca(OH)_2$ did not effectively reduce the dry matter losses (DML) of poplar wood chips, neither after short- (35 d) nor long-term storage (120 d) at industrial scale. As

expected, the highest DML were reached in the second storage phase, showing mean values of less than 10% in all three piles (0%, 1.5%, 3% Ca(OH)$_2$). In practice, wood chips are often stored for more than four months until they are consumed by a bioenergy facility. However, we considered a storage period of 120 d as representative for long-term storage as recent studies revealed that the strongest increase in DML occurs almost entirely within the first three to four months of storage, showing hardly any further increase thereafter (Lenz et al., 2015; Pecenka et al., 2018). Even though woody biomass was not successfully preserved by the addition of Ca(OH)$_2$, calcium-based additives can positively reduce ash slagging during biomass combustion (Xiong et al., 2008, 2017), which should be tested in this context. DML and decomposition of wood chips are mainly linked to microbial activity, however, these habitats (wood chip energy piles) have so far been poorly investigated from an ecological and applied microbiological point of view, in particular with cultivation-independent methods. Hence, the aim of this study was not only to determine the effect of Ca (OH)$_2$ addition on the microbial community, but also to identify potential key players in poplar wood degradation using high throughput next-generation sequencing based on whole community DNA. However, this does not allow the distinction between active and inactive (e.g. spores) and/or living from dead members of the microbial communities which should be kept in mind. Another factor that might have influenced the results from next generation sequencing is intraspecies variance. Especially fungi, but also bacteria can code different ITS and 16S genes, respectively, in their genomes (Schoch et al., 2012; Větrovský and Baldrian, 2013). As a result, the total number of ASVs could potentially overestimate diversity. However, in our study we focused on changes in ASV richness between piles, which will be equally affected. As differences in cluster thresholds and between taxonomic units (OTUs) and ASVs have been shown to give similar results (Glassman and Martiny, 2018), allelic variation is unlikely to have affected our conclusions.

Both bacterial and fungal communities were driven by the effects of storage time and Ca(OH)$_2$ addition, albeit the factor time was by far the most influencing. Wood chipping increases the surface area of the woody material and the accessibility of easily degradable compounds, thereby leading to a high microbial activity and rapid increase in temperature already within the first days of storage (Ferrero et al., 2011; Whittaker et al., 2018; Idler et al., 2019). Indeed, temperatures exceeding 60 °C were measured in the pile without any amendment as well as in the piles supplemented with 1.5% and 3% Ca(OH)$_2$, which is in agreement with previous studies (Barontini et al., 2014; Lenz et al., 2015; Pari et al., 2015; Pecenka et al., 2018). We hypothesize that the time-dependent differences observed in the microbial communities were mainly a result of temperature differences, whereas nutrient availability and other factors might have played a role. However, in the piles supplemented with Ca(OH)$_2$, maximum temperatures were reached on average two days earlier (4 d of storage) compared to the pile without any amendment (6 d of storage). The chemical reaction of Ca(OH)$_2$ with atmospheric CO$_2$ leads to the production of CaCO$_3$, water and heat (Moorehead, 1986), which is not only supposed to accelerate the rise in pile temperature, but also to increase maximum temperatures as it was observed in the piles with Ca(OH)$_2$ addition. Additionally, natural drying of wood chips was delayed in the piles supplemented with the alkaline additive, which is probably ascribed to the same chemical reaction as water is released.

Supporting the hypothesis of Baas-Becking (1934) ("Everything is everywhere, but the environment selects"), 89% of the bacterial and 97% of the fungal amplicon sequence variants (ASVs) observed at storage intake were not detected in wood chips stored for 35 d, indicating an almost entire shift in these communities. The high diversity commonly found in natural environments was probably adapted to mainly two harsh selective pressures: Once the easily degradable parts of wood chips were removed, the more recalcitrant components (cellulose, hemicellulose, lignin) remained and their decomposition was primarily attributed to a defined microbial consortium specialized in

wood degradation (Ferrero et al., 2011). Moreover, high temperatures are very selective for the majority of environmental microorganisms. Especially at temperatures >60 °C, the degradation of the woody material is a self-limiting system debilitating the microbial community. This process is known from similar environments such as composts and it is often referred to as "microbial suicide" (Finstein et al., 1980; MacGregor et al., 1981; de Bertoldi et al., 1983). Numerous fungal families, for example, were solely detected at storage intake, but not in stored wood chips, emphasizing their inactivation by heat, as fungi are known to be far more thermolabile than prokaryotes (de Oliveira et al., 2015).

Among fungi, *Aspergillus fumigatus* is thermotolerant and thus, the major air-borne fungal pathogen causing several forms of diseases in humans when inhaling its spores (McCormick et al., 2010; Kwon-Chung and Sugui, 2013). Regardless of the Ca(OH)$_2$ concentration, this taxon was detected not only at storage intake but also in stored wood chips, supporting its persistence at high temperatures during the initial stages of storage. Previous studies identified *A. fumigatus* as commonly found mold during the storage of poplar wood chips (Horváth et al., 2012; Suchomel et al., 2014) and this species is one main reason for special safety regulations on storage sites. However, genomic studies underlined the relevance *of A. fumigatus* for wood decomposition as it revealed several genes involved in the degradation of cellulose, hemicellulose and lignin (Tekaia and Latgé, 2005). Likewise, the thermophilic fungus *Thermomyces lanuginosus* has been commonly isolated from environments comparable to wood chip piles and is known to produce xylanases, enzymes involved in the degradation of hemicellulose (Maheshwari et al., 2000; Singh et al., 2003; de Oliveira et al., 2015). Although this fungus is known to have optimum growth temperatures between 45 and 50 °C (Maheshwari et al., 2000), *T. lanuginosus* dominated fungal communities of both short- and long-term stored wood chips showing respective pile temperatures of approximately 30 °C. Maheshwari et al. (2000) suggested that high temperatures are crucial for spore germination of thermophilic fungi, while the maintenance of high temperatures for sustaining mycelial growth may not be critical as several thermophilic species show growth at mesophilic temperatures. Hence, it is assumed that *T. lanuginosus* and probably other thermophilic microorganisms were already part of the autochthonous microbial community at storage intake, whereas spore germination and thereof mycelial growth were only favored by the high temperatures at the initial stages of storage. Indeed, just a small number of reads was affiliated to this taxon at storage intake being not representative for the whole sample group as they were removed when data were filtered. Moreover, species affiliated to the family Chaetomiaceae were among the most abundant fungal species in stored wood chips irrespective of Ca (OH)$_2$ concentration. Even though these taxa could not be assigned at species level, several members of this family tolerate high temperatures (de Oliveira et al., 2015) and are classified as soft-rot fungi, which are involved in the degradation of all major woody components (Maheshwari et al., 2000; Tuomela et al., 2000), emphasizing their role as potential key players during wood decomposition.

Although the degradation of woody biomass is mainly associated with fungal activity (de Boer et al., 2005; Noll and Jirjis, 2012), many of the bacterial species dominating short- and long-term stored wood chips are known to degrade lignocellulosic biomass. Members of the genus *Streptomyces* have been commonly isolated from similar environments to wood chip piles and they are regarded as metabolically versatile and capable of degrading cellulose, hemicellulose and lignin (Antai and Crawford, 1981; Tuomela et al., 2000; Saini et al., 2015). Species annotated as *Actinomadura*, *Thermoactinomyces* and Micromonosporaceae are known to degrade the carbohydrate components of the woody material (Pérez et al., 2002; Jiao et al., 2015; Saini et al., 2015; Yin et al., 2018), whereas previous studies described the potential of strains affiliated to the genera *Brevibacillus* and *Ochrobactrum* to produce also ligninolytic enzymes (Vargas-García et al., 2007; Hooda et al., 2018; Tsegaye et al., 2018; Xu et al., 2018). Similar to the fungal

species dominating the microbial community in stored wood chips, most of these taxa are known to be either thermotolerant, thermophilic or to produce heat-resistant spores (Galperin, 2013; Carrillo and Beníte-z-Ahrendts, 2014; Jiao et al., 2015; Shivlata and Satyanarayana, 2015). Hence, it is suggested that both features the ability to degrade ligno-cellulosic materials and the tolerance of increasing temperatures were necessary for the establishment of stable communities in stored wood chips. Affirming these results, species belonging to the orders Enter-obacteriales and Pseudomonadales dominated the bacterial community at storage intake, however, their abundances dramatically decreased during storage, which is probably due to their thermolability rather than their inability to degrade woody components. Many of these taxa have been reported to degrade lignocellulosic biomass (Jones et al., 2018; Xu et al., 2018).

The effects of Ca(OH)$_2$ addition on the microbial community were assessed after a storage time of 120 d. Unexpectedly, none of the fungal ASVs detected after long-term storage of poplar wood chips were significantly influenced by the amendment of Ca(OH)$_2$, emphasizing their tolerance of neutral to slightly alkaline environments. Indeed, among the most abundant fungal species, the pH optimum of *T. lanuginosus*, for example, has been reported to be near neutral (Rosenberg, 1975) and *A. fumigatus* is regarded to grow under both acidic and slightly alkaline conditions (Kwon-Chung and Sugui, 2013; Pugliese et al., 2018). Regarding the more versatile bacterial commu-nity, several ASVs were detected to be either promoted or inhibited by the addition of different Ca(OH)$_2$ concentrations. If these species were correlated to pH, significant dependencies were observed in almost all cases, confirming the main effect of Ca(OH)$_2$ on elevating the pH of wood chips. As expected, positive correlations with pH were observed if highest abundances were reached in the presence of Ca(OH)$_2$ and vice versa. The taxonomic assignment of bacterial ASVs was executed at genus level of which most of them are composed of widely distributed species tolerating broad ranges of pH and therefore, the potential to connect our results with those of previous studies is limited. Exem-plarily, members of the genus *Streptomyces* are supposed to prefer a neutral to alkaline pH, however, they occur in a broad pH range depending on the availability of nutrients (Kontro et al., 2005). We identified one ASV annotated as *Streptomyces* to thrive in the absence of Ca(OH)$_2$, whereas another ASV assigned to this taxon reached its highest abundance in the pile supplemented with 3% Ca(OH)$_2$, supporting the diversity of this genus. Interestingly, ASVs annotated as N-fixing Rhi-zobiaceae were significantly more abundant in the high Ca(OH)$_2$ pile. It is likely that N-fixing bacteria provide an additional N-source to fungi. In fact, especially soft-rot fungi need high nutrient amounts in order to efficiently decompose deadwood (Daniel and Nilsson, 1998). However, for lime stabilization of biosolids, elevating pH to 12 is suggested for effective inactivation and elimination of microorganisms (Bean et al., 2007; Williford et al., 2007). As a consequence thereof, we conclude that the elevation of pH in the piles supplemented with Ca(OH)$_2$ was insuf-ficient to successfully reduce microbial growth and thereof DML compared to the pile without any amendment. Decreases in the C:N ratio are regarded as alternative indicators of wood chips degradation due to the relative consumption of C and N during microbial decomposition (Whittaker et al., 2018). However, C:N ratios of long-term stored wood chips did not significantly vary among the three different piles (0%, 1.5% and 3% Ca(OH)$_2$), corroborating the negligible effects of Ca(OH)$_2$ addition on wood degrading microbes.

5. Conclusion

This study was the first of its kind monitoring both bacterial and fungal communities in industrial-scale poplar (*Populus canadensis*) wood chip piles under different storage conditions (0%, 1.5% and 3% Ca(OH)$_2$ addition) by using next-generation sequencing. Even though the addi-tion of different Ca(OH)$_2$ concentrations changed physicochemical properties of the woody material, the resulting temperature differences

and elevation of pH were insufficient for substantial reduction of mi-crobial decomposition and preservation of woody biomass. However, to which extent the composition of the woody material (e.g. hardwood, softwood) influences the efficacy of Ca(OH)$_2$ as alkaline stabilization agent should be clarified in further studies. Nevertheless, our findings provide a comprehensive insight into bacterial and fungal communities of wood chips, their compositions and dynamics during a storage period of 120 d. Whereas the microbiota of freshly harvested and stored wood chips was almost completely different, both short- and long-term stored wood chips were dominated by a similar consortium of lignocellulolytic and thermotolerant microorganisms. As a result, shifts in the microbial communities were primarily ascribed to the development of pile tem-perature as well as the availability of nutrients.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2020.105133.

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