DNA quantification of basidiomycetous fungi during storage of logging residues

The demand for bioenergy caused an increased use of logging residues, branches and treetops that were previously left on the ground after harvesting. Residues are stored outdoors in piles and it is unclear to what extent fungal decomposition causes dry matter loss. Our objective was to quantify the amount of wood degrading fungi during storage using quantitative real-time PCR (qPCR) to detect basidiomycetous DNA in logging residues, a novel approach in this field. We found that the qPCR method was accurate in quantifying the fungal DNA during storage. As the moisture content of the piled logging residues decreased during the storage period, the fungal DNA content also decreased. Scots pine residues contained more fungal DNA than residues from Norway spruce. Loose piles had generally more fungal DNA than bundled ones. In conclusion, fungal degradation of wood was not the main cause of dry matter loss.

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1. Introduction

Logging residues, also known as forest slash, are branches and tops left on the forest site after logging. In Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.) residues correspond to about 55 and 20% of the stem volume, respectively (Hakkila 1991). In the past the logging residues were uneconomical to gather and were left in the forest. With the increased need to utilize all renewable sources of energy, this lignocellulosic biomass provides a new potential as a fuel, e.g. chips for waterborne heating. Because loose logging residues are bulky to handle, they are sometimes compressed into bundles and piled for easier handling and transportation (Johansson et al. 2006). To promote drying of the material, piles are left in the forest or at the roadside for variable periods of time until further processing to final biofuel. While storage is used as a method to reduce moisture and thus makes the material better suited as fuel, the material also loses dry matter. Filbakk et al. (2011) found that the total dry matter loss in fresh logging residues of Scots pine and Norway spruce was 1-3 % per month, while Flinkman & Thörnquist (1986) reported a monthly dry matter loss of 0.2 % of fresh and 1.2 % of old, bundled logging residues.

The initial dry matter loss can be attributed to so-called transpiration drying – loss of moisture, when transpiration continues through the attached foliage and cut surfaces for some time even after the trees have been harvested. The transpiration drying causes the foliage and dry twigs to fall off and thus contributes to the total dry matter loss. The total dry matter loss is determined usually as a difference in initial and final dry biomass (Andersson et al. 2002). Jirjis & Norden (2005) estimated that needle loss caused 5.8% dry matter loss after 5.5 months of storage of fresh logging residues in bundles but in pre-stored logging residues, where needles have dropped off before pile-construction, the loss was less than 1 %. In a Scandinavian review of storage experiments with chipped logging residues the mean dry matter loss was 1-5% per one month of storage (Gislerud 1979). Although drop of needles and twigs is increasing the physical dry matter loss, it is beneficial because the high nutrient content of this material results in high ash content, which lowers the combustion efficiency. However, the deterioration of lignocellulosic material due to microorganisms needs to be minimized because this type of dry matter loss is economically unprofitable.

Especially fungi are the main degraders of woody materials (Dix & Webster 1995). However, to our knowledge, the fungal contribution to dry matter loss during storage of unprocessed logging residues, tops and twigs, has not been quantified.

For fungal growth the moisture content and temperature are essential factors. Fungal decomposers are divided into three functional groups according to their substrate utilization pattern: white-, brown- and soft-rot fungi (Cooke & Rayner 1984). White-rot fungi are mainly basidiomycetes and the only organisms known to be able to effectively utilize the lignin, cellulose and hemicellulose in various proportions (Cooke & Rayner 1984). Brown-rot fungi, which appear to be exclusively basidiomycetes, utilize cellulose and hemicellulose, leaving the

modified lignin in place (Cooke & Rayner 1984). Soft rot decay by ascomycetes and mitotic fungi primarily occurs under conditions where the growth of the generally more active and competitive basidiomycetes is retarded (e.g. high moisture, low aeration). The decay caused by soft rot fungi is generally slower than decay caused by basidiomycetes. Basidiomycetes are likely the fungal group most responsible for the logging residue degradation (Tuomela et al. 2000).

To detect fungi in materials, traditionally the classical techniques of spore counting, cultivating and microscopy were used. Flinkman & Thörnquist (1986) studied storage of bundled, unlimbed pulpwood and logging residues and found that the occurrence of microfungi in bundled material did not differ to any particular extent from loose material after eight months. They also noted that assortments with relatively large proportions of needles and bark appeared to provide the most favourable substrates for fungi. Jirjis & Lehtikangas (1993) studied fuel quality and dry matter loss during storage of logging residues in a pile. They found a general increase in spore count during approximately one year of storage and also an increase in viable spores. In another study by Lehtikangas & Jiris (1995) of logging residues in covered piles the spore count at the end of the storage period (one year) was lower than at sampling after 7 months of storage, while the number of viable spores slightly increased. Spore counting does not discriminate between decay fungi and other fungal spores without additional isolation and identification in the lab. However, the development of DNA-based PCR (Polymerase Chain Reaction) and taxon-specific primers has provided a range of new possibilities. For example Piskur et al. (2011) used PCR-DGGE method to analyze the fungal communities in degraded wood chips. Quantitative real-time PCR (qPCR) has proven to be a useful tool for the detection of plant pathogenic fungi and bacteria (Hietala et al. 2009; Salm & Geider 2004; Schaad & Frederick 2002; Schena et al. 2006; Vandroemme et al. 2008), but also wood deteriorating fungi (Eikenes et al. 2005; Pilgård et al. 2011; Pilgård et al. 2010). The method is highly sensitive, specific and rapid, with the added capacity for quantification. To our knowledge, the qPCR approach has not so far been used to indirectly quantify the loss of biomass in logging residues for bioenergy utilization due to wood deteriorating fungi.

The forestry practice of using the logging residues as a fuel chips is relatively recent and there is little available documentation on how microbial processes in stored piles may influence the final quality of the material as a fuel. Because the fungi degrade the wood and thus use up its energy, the amount of basidiomycetous DNA (indicative of the amount of fungal biomass) is correlated with the mass loss (Eikenes et al. 2005). Hence, we make the following assumption: the more fungal DNA measured, the more dry matter loss. The dry matter loss caused by fungi during storage needs to be reduced as much as possible. By understanding the fungal colonization in stored logging residues better, this knowledge can be used to 1) better understand the potential effect on dry matter loss related to fungal degradation and 2) optimize storage methods further.

This paper is based on the same samples as described in Filbakk et al. (2011). They modelled moisture content and dry matter loss during storage of logging residues. The first aim of this study was to implement quantitative real-time PCR (qPCR) as a novel technique to quantify the

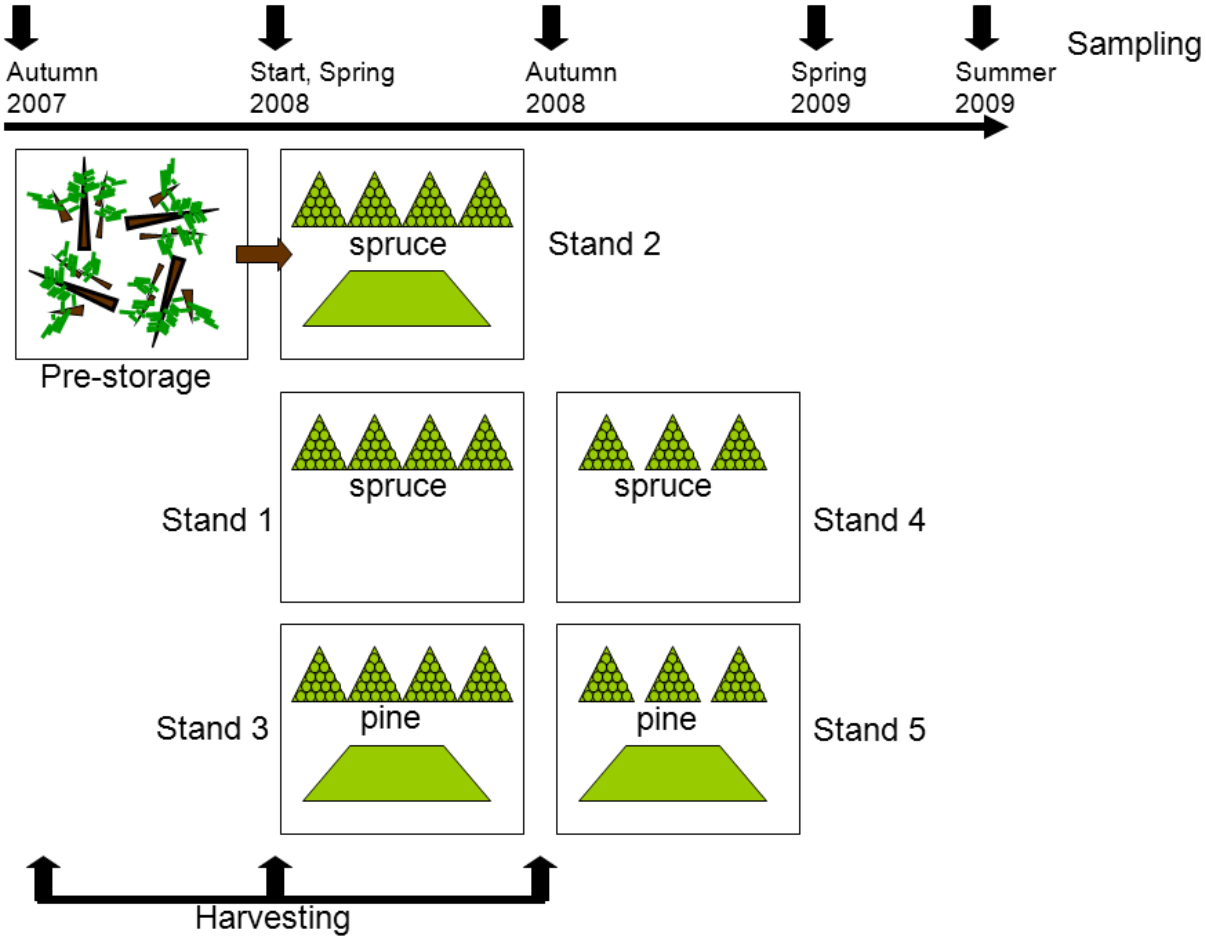
fungi in logging residues. The second aim was to find out whether the storage conditions of forest residues promote fungal growth to such a degree that it will significantly impair the final dry mass and fuel quality of the logging residues.

2. Experimental Section

2.1. Experimental setup and sample taking

The detailed description of the experimental setup and sampling is given in Filbakk et al. (2011). Briefly, this study was carried out with residues originating from five different harvesting sites, all located close to Braskereidfoss (60º 62'N/ 12 º 02'E), Norway. Three stands were predominantly Norway spruce (*Picea abies* (L.) Karst.) and two were dominated by Scots pine (*Pinus sylvestris* L.), all 70-100 years old. The experimental setup is illustrated in Figure 1. Stand 2 was harvested in the autumn 2007 and logging residues were pre-stored; left lying on the clear-cut site until spring 2008 when piles were constructed. Stands 1 and 3 were harvested in spring 2008, and Stands 4 and 5 were harvested in autumn 2008. After each harvesting, the residues were stored in two types of piles, either in pyramid-like piles consisting of bundled residue material or loose piles consisting of unbundled material (Fig. 1). To protect the piles from precipitation, each pile was covered. Samples from piles were taken before storage (Start), then in spring 2008, autumn 2008, spring 2009, and summer 2009 (Fig. 1). At each sampling five replicates (1-2 kg) were taken from about 1000 kg of chipped material from each treatment. To obtain homogenous consistency, samples were grinded in three steps (see below, 2.2). All samples were analyzed for moisture content by the oven drying method at 103˚C (CEN/TS-14774-1 2004), and for calorific value (CEN/TS-14918 2005). The total dry matter loss in bundled piles was calculated as the difference between initial and final biomass (Filbakk et al. 2011).

Figure 1. Experimental setup. After the first harvesting (felling of trees) the material was pre-stored by being left on the clear-cut during the winter. In the second and third harvesting periods piles of bundled (triangle symbol) and loose residues (trapezoid symbol) were constructed. At each sampling one bundled pile and a quarter of a loose pile were analyzed.



2.2. DNA extraction

From each chipped sample, about 0.4 kg was randomly taken, dried at 50°C for 24 hours, and grinded first coarsely (Retsch Mühle grinder, 5 mm mesh, Retsch Gmbh, Germany) and then finely (IKA Werke MF 10 basic grinder, 0.5 mm mesh, IKA®-Werke Gmbh & Co., Germany). The same sample batch was used for analyses by Filbakk et al. (Filbakk et al. 2011). About 80 mg of finely grinded material was milled to powder-consistency in liquid nitrogen using a Retsch mixer mill (MM 300, Retsch Gmbh, Germany). Aliquots of 20 mg were prepared from powdered material for each treatment and total DNA was isolated using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The protocol provided by the manufacturer was followed. To account for the variation in DNA extractability in the environmental samples and to normalize for this variation, 5 ng of an external reference DNA, pGEM plasmid (pGEM-3Z Vector, Promega, Madison, WI),

was added to each sample upon start of DNA extraction (Hietala et al. 2009). The resulting total DNA extracted was eluted in 50 µl of buffer AE and stored at -80 ˚C until processed by qPCR.

2.3. Primer selection

The primers were based on prior findings by Vilgalys & Hester (1990) and Fierer et al. (2005) with minor modification to improve the number of basidiomycetes amplified. As a forward primer the 5.8sr TCGATGAAGAACGCAGCG primer was used (Fierer et al. 2005; Vilgalys & Hester 1990) and as a reverse primer we selected a 2 nucleotide truncated ITS4-X primer CAG GAG ACT TGT ACA CGG TCC as it amplifies a large set of basidiomycete species. Primers for the internal pGEM control for extractability were selected as previously described by Coyne et al. (2005) and Pilgård et al. (2010).

To test the primer specificity for basidiomycetes, pure cultures of organisms representing the target DNA (basidiomycetes) were used as positive controls (*Armillaria borealis* 2005-713/2, *Fomitopsis pinicola* 1946-755/2, *Coniophora puteana* 1982-97/3 and *Schizophyllum commune* 1956-1236/1), and as negative controls non-target DNA (aseptic spruce seedling roots, *Trichoderma* sp. 1959-1919/471, *Penicillium* sp. 2007-161/14/3 and *Cladosporium cladosporoides* 1967-149/11), all fungi obtained from the Norwegian Forest and Landscape Institute's fungal culture collection (http://www.skogforsk.no/skogpatologi/database/searchform.cfm). Fungi were grown in petri dishes on cellophane over malt agar medium, incubated at 25 °C. Also, samples with known dual mixtures of basidiomycete DNA and host tree (*Serpula*-degraded and *Trametes*-degraded pine wood) together with pGEM were grown to verify the specificity of the primers used. The DNA from all organisms was extracted and PCR-amplified using the selected primer pair. The presence of the amplified product was visualised on the agarose gel.

2.4. qPCR conditions

The real-time qPCR detection of basidiomycete DNA was performed using SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and the reference pGEM was detected with TaqMan Universal PCR Master Mix (Applied Biosystems, #4304437).

The internal standard pGEM for calibrating DNA extractability was quantified as described by Coyne et al. (2005). The pGEM standard curve was prepared from serial diluted samples containing 0.066 to 0.000066 ng of pGEM DNA giving a standard curve of $y = 2.7371 – 0.2642x$ (x is the Cq value and y the logarithmic amount of DNA).

A standard curve was prepared from DNA isolated from our target DNA, basidiomycetous fungi (*A. borealis*, *F. pinicola*, *C. puteana* and *S. commune*) grown on malt extract agar, using serial diluted samples of 0.03 to 0.0003 ng of DNA giving a standard curve of $y = 3.0922 – 0.176x$ (x is the Cq value and y the logarithmic amount of DNA).

For detection and quantification of basidiomycetous DNA the primer concentrations were 60 nM. For detection and quantification of pGEM plasmid, the primer concentrations were 300 nM

and probe 300 nM. As a template, 3 µl of sample extracted total DNA solution was used for each reaction. The real-time detection was performed with an ABI PRISM 7700 (Applied Biosystems). After amplification the data were analyzed and plotted (fluorescence vs. cycle number) using the Sequence Detection System, version 1.7a, Software Package (Applied Biosystems). The extent of amplification was calculated as a mean Cq value of 2 replicates for each sample. All PCR reactions were performed in singleplex conditions under standard PCR cycling parameters. Undiluted, 10x, 100x, 1000x and 10000x diluted experimental sample concentrations were tested, but 100x dilution gave the best results and will be presented.

2.5. Statistical analysis

We analyzed the amounts of fungal DNA in logging residues from five forest stands as dependent parameter against the following independent categorical parameters: tree species, harvesting times, storage, within bundle placement, storage time. Precipitation, moisture, bundling time, mean temperature, dry matter loss and calorific value were continuous parameters in the model (Tables 1 and 2). The values for fungal DNA were approximately normally distributed. To test whether the parameters had significant effects on the amount of fungal DNA, we used analysis of variance (ANOVA) for all categorical parameters and linear regression for all continuous parameters. In addition we tested interaction among wood moisture and storage method. First we tested whether pre-storage, leaving the logging residues on the harvesting site during the winter, had a significant effect on the fungal DNA between Stands 1 and 2. Because it did not, we included all five stands in the same model. Means in categorical parameters were compared by Tukey-Kramer HSD test at $P<0.05$). In all cases, a null hypothesis was rejected at the 5% level of significance. All statistics were done using JMP (SAS Institute Inc., Cary, NC, USA).

Table 1. Description of parameters used in the study.

Parameter	Type	Explanation	Unit
Harvesting time	categorical	Season when the trees were cut (harvested)	Start, spring, autumn
Storage time	categorical	Days after the piles were made	Days
Precipitation	continuous	Mean precipitation since last sampling	Mm
Temperature	continuous	Mean temperature within piles	°C
Wood moisture	continuous	Moisture content measured in the sampled material	%
Tree species	categorical	Dominant tree species in the logging residue	Spruce, pine
Storage method	categorical	Piles made of loose or bundled forest residues	Start, loose, bundles
Calorific value	continuous	The amount of energy per kg given off when burnt	MJ/kg
Total dry matter loss	continuous	Total loss of dry matter during the storage, including fall off	%

		needles, twigs and microbial degradation	
Placement	categorical	Location of bundles within piles	Top, bottom, middle
Pre-storage	categorical	Leaving the logging residue spread on the clear-cut site after harvesting, throughout the winter	Pre-storage, piles

3. Results and Discussion

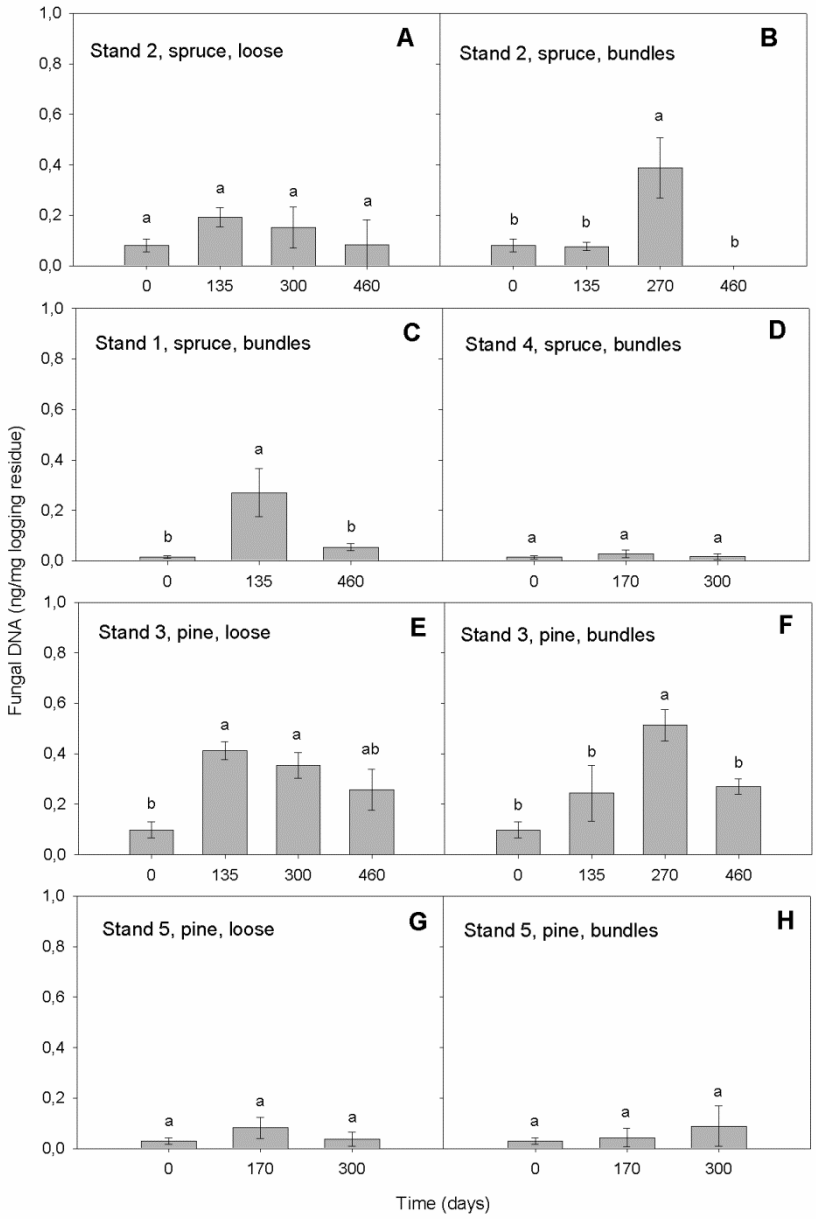
3.1. qPCR assay

The real-time qPCR primers used in our assay were specific for basidiomycetous DNA, while the non-target DNA used as negative controls (host tree DNA, bacterial and non-basidiomycetous fungi) was not amplified under the conditions used. Likewise, the primers used for the calibration of DNA extractability between samples amplified only the pGEM plasmid used as an internal control in these studies (data not shown). The qPCR can detect down to one fungal genome (one fungal cell) corresponding to the lowest point on our standard curve ((~0.0003ng).

Our qPCR assay was sensitive and able to quantify fungal DNA in logging residues. It detected in the range of 0.01 – 0.69 ng basidiomycete DNA/mg in chipped logging residue (Fig. 2). That the most elevated amounts of DNA were detected after the highest precipitation event promoting fungal growth (see below, 3.2.1 and 3.2.3) is an indicator that the sampling was representative and that the method was sufficiently sensitive. Also, the same general pattern was found in all stands: The lowest DNA value was detected at the start of the storage period, followed by increase in the wetter period in spring 2009, followed by a decrease at the dryer end of the storage, suggesting that our detected values may reflect the true situation. Our basidiomycete DNA values were considerably higher than those found by Pilgård et al. (2011) in Scots pine heartwood, preservative treated and furfurylated stakes in a soil contact (EN 252, 1989), when using the same assay. However, the substrates and exposure situation in these two studies were very different; Scots pine samples were treated to hinder the fungal growth in soil contact by Pilgård et al. (2011), whereas in this work the forest residues were untreated and thus likely exposed to higher colonization potential by fungi, which can explain our higher DNA values.

Although the qPCR assays are highly sensitive with high resolution and correlation to mass loss at the early stages of decay, they may lack accuracy in advanced decay stages due to substrate depletion (Eikenes et al. 2005). We did not have this challenge with our material since the level of decay was only in its initial stages of colonization and thus substrate availability was plentiful for further fungal growth.

Figure 2. Amount of fungal DNA (ng/mg logging residue) detected in logging residue after increasing storage time (days) in all five stands, in loose and bundled piles of spruce and pine (n=133). Standard errors are marked as vertical lines and means are compared by Tukey-Kramer test at $P<0.05$, where different letters denote significant differences.



3.2. Fungal DNA and storage conditions

The statistical model we used included data from all stands and explained 68% of the variability in the data (Table 2, n=133, R^2=0, 68). Of all the parameters we tested, only harvesting time, storage time, precipitation, temperature, wood moisture content, tree species and storage method significantly affected the amount of basidiomycete DNA detected in the logging residues (Table 2). In addition, wood moisture*storage method contributed significantly.

Table 2. Test statistics and parameter estimates of the model.

	Variance components:
R^2	0.68
R^2 adj	0.65
RMSE	0.10
N	133

Parameter estimates for the covariates in the model and P-values from effect tests:

	Parameter estimate	P-values
Intercept	0.3350	0.0024*
Storage time	-0.0004	<.0001*
Precipitation mm	0.0333	<.0001*
Storage method	-	<.0001*
Mean temperature	-0.0054	0.0040*
Harvesting time	-	0.0061*
Tree species	-	0.0091*
Wood moisture*Storage method	-	0.0397*
Wood moisture	-0.0045	0.0398*

Parameter estimates for categorical variables and Tukey-Kramer (T-K) 0.05 tests on LS means:

	Parameter estimate	T-K
Harvesting time [Autumn 2007]	0.0301	A
Harvesting time [Spring 2008]	0.0218	A
Harvesting time [Autumn 2008]	-0.0519	B
Tree species [pine]	0.0351	A
Tree species [spruce]	-0.0351	B
Storage method [Start]	-0.1393	C
Storage method [Bundle]	0.0334	B
Storage method [Loose]	0.1059	A
Wood moisture*Storage method [Start]	0.0079	-
Wood moisture*Storage method [Bundle]	-0.0026	-
Wood moisture*Storage method [Loose]	-0.0053	-

*Significance level 0.05

3.2.1. Variation in fungal DNA over time

In the model, the storage time affected the fungal DNA amount significantly ($p<0.0001$). Generally, the amount of fungal DNA decreased at the end of the storage (Fig. 2, 3). In all stands and pile types we detected the lowest DNA values at the start, slightly rising during the storage

and decreasing at the end of the study (Fig. 2). The transient DNA increase was significant for the combinations illustrated in Fig. 2B, C, E, and F.

The highest fungal DNA content was detected in bundles after 270 days, in spring 2009, in a Norway spruce stand (n=5, mean 0.39ng DNA, Fig. 2B) and in a Scots pine stand (n=5, mean 0.51 ng DNA, Fig. 2F). Both peaks coincided with the highest precipitation (9 mm) measured during the entire study period, almost 5-fold higher than the mean precipitation.

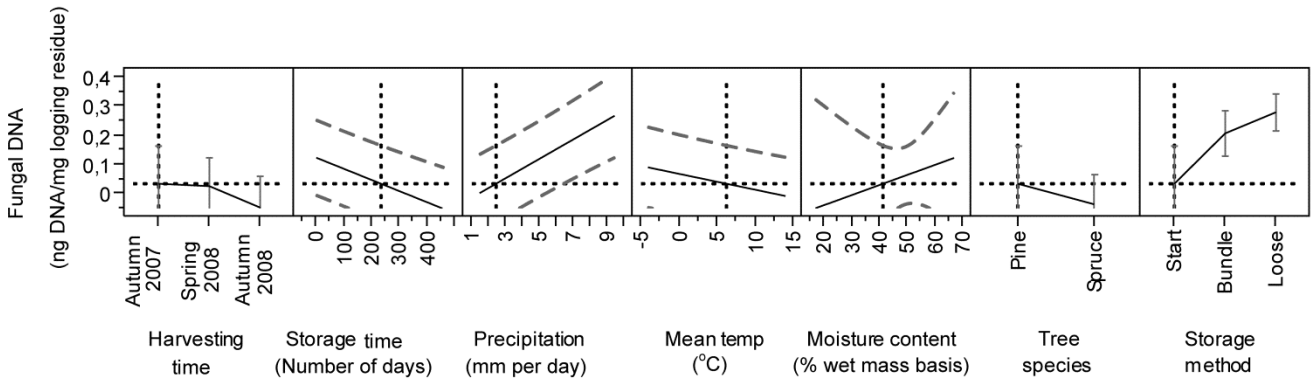
The general decline in amount of fungal DNA with time may be due to transpiration drying, as transpiration continues from foliage or open wood surfaces after harvesting (Andersson et al. 2002). Indeed, such a decline in moisture has already previously been documented in this material by Filbakk et al. (2011).

Material from Stands 4 and 5, with the lowest DNA values, was harvested and piled in autumn 2008 and had the shortest storage period. It is likely that the conditions were not conducive for fungal development during most of the storage period, since basidiomycete fungi have restricted growth at low temperatures.

3.2.2. Seasonal effects

The model showed a significant contribution of the harvesting time (time of felling), to the amount of fungal DNA (Table 2). We detected significantly lower start fungal DNA values from material harvested in autumn 2008 than in autumn 2007 and spring 2008 (Table 2, Figure 3). The moisture content in the stored biomass tends to increase in late autumn and winter in the Nordic climate (Nurmi 1999; Pettersson & Nordfjell 2007). Although studies by Nurmi & Hillebrand (2007) showed that harvesting in spring gave better conditions for drying, thus less favorable conditions for fungi, we cannot confirm this observation as we did not have a sufficient number of harvesting replications.

Figure 3. Estimates of fungal DNA in logging residues (ng DNA/mg logging residue) in relation to harvesting time, storage time, precipitation, temperature, moisture content, tree species and storage method.



3.2.3. Moisture

In the model wood moisture ($p = 0.0398$), precipitation ($p < 0.001$) and wood moisture*storage method ($p = 0.0397$) contributed significantly in explaining basidiomycete DNA content (Table 2). The prediction profiles illustrate that fungal DNA increased with precipitation and wood moisture content. We found highest values of fungal DNA in material with moisture 30-35% (Fig. 3). In general, as expected, the moisture content decreased during storage for all materials, in Stand 2 from 55 to 50%, Stands 1 and 3 from 45% to 30%, Stand 4 from 45 to 40% and Stand 5 from 55 to 40% (Filbakk et al. 2011). The average moisture content reduction was 12% larger in summer than in winter, the relative air humidity in the piles was about 100 % and the temperature in the piles closely followed the ambient temperature, due to fairly small piles, never exceeding 19°C (Filbakk et al. 2011). The significant interaction of moisture*storage method may be because the „Start“ was included in our study as one of the storage methods (Table 2, Fig. 3) and the moisture was highest at this point.

Our results indicate that the moisture content in the piles was sufficient for fungal growth, never below 20%. When wood moisture content drops below 20%, water is retained only in the cell wall micropores and fungi cannot utilize it because their cell wall degrading enzymes are too large to enter the micropores (Griffin 1977). However, fungi can survive in a dormant state and rewetting will revive them (Findlay 1950). In general, optimal moisture for growth of the majority of wood decaying basidiomycetes is in the range between 40 and 80% (Eaton & Hale 1993).

Indeed, the high amount of precipitation (9 mm) in spring 2009, after 270 days of storage, was associated with higher fungal DNA values (Fig. 2B, F). The temporary peak DNA values related to peak moisture in the material (Filbakk et al. 2011) further indicate that even a short period of high precipitation may increase the fungal growth.

3.2.4. Tree species

Tree species contributed significantly ($p=0.0091$) to the amount of fungal DNA in logging residues (Table 2). The Tukey-Kramer test showed a significant difference between Scots pine and Norway spruce (Table 2). We found significantly more fungal DNA in pine-dominated residues ($n =64$, mean 0.19 ng DNA) than in spruce-dominated ones ($n =69$, mean 0.10 ng DNA), and there was larger variation in values in pine than in spruce residues (Figs. 2, 3).

The significant differences between the tree species were mainly due to the high DNA values we detected in pine stand 3. Pine stand 5 had very low values. Also, because the spruce has a higher percentage of small, flexible branches with needles the bundles are usually denser than pine bundles, with more moisture and less airflow.

The high variability among the fungal DNA found at the five different stands, with extremely high values in pine stand 3 (mean 0.5 ng DNA) and correspondingly low values at spruce stand 4 (mean 0.02 ng DNA), probably contributed to the significant differences among the tree species.

3.2.5. Storage method

The storage methods alone ($p<0.0001$) and also in combination with moisture ($p=0.0397$) contributed significantly in explaining the amount of fungal DNA in logging residues (Table 2). We found significant differences in fungal DNA content among the three storage methods. In all stands the amount of fungi was, as expected, always lowest at start, before bundling or loose pile construction (n=20, mean 0.04 ng DNA). Compared to start, the DNA amount was significantly higher in bundles (n=65, mean 0.16 ng DNA) and highest in loose residues (n=48, mean 18 ng DNA).

Only minor differences were detected in the moisture content between bundles and loose piles (Filbakk et al. 2011). Hence the consistence of loose piles may have created microclimate with more air flow, thus more conducive to fungal development than in the compacted bundles.

We did not find any significant differences in fungal DNA amount related to material location within the pile. Filbakk et al. (2011) found the highest material moisture in the middle of the pile, however the moisture content in this study was always within the favorable range for fungal growth (Eaton & Hale 1993).

When comparing bundled spruce material, with or without pre-storing in Stands 1 and 2, we found that the mean DNA content was similar, 0.11 and 0.13 ng DNA respectively (n=42, Fig. 2B,C). This may imply that the pre-storage on the harvesting site during the winter, before piling, does not increase the decay rate of the logging residues.

3.2.6. Dry matter loss

There was no significant effect of fungal DNA amount on dry matter loss. We detected dry weight loss of about 15-30% in bundles during the entire storage period (Filbakk et al. 2011). This corresponded to 3% and less than 1% loss per month for Norway spruce and Scots pine, respectively (Filbakk et al. 2011). Eikenes et al. (2005) showed that in laboratory conditions the amount of 1 ng/mg DNA of white-rot fungus *Trametes versicolor*, quantified by qPCR method, corresponded to about 25% mass loss of birch wood after 4-20 weeks of decay. This substantial mass loss reflects a decay potential of *T. versicolor* under optimal growing conditions. However, these data cannot be used as a conversion of our fungal DNA values to estimate the corresponding mass loss in our experiment as the growing substrate, organisms and physical conditions were completely different.

While in our study the total dry matter loss increased over time (Filbakk et al. 2011), the fungal DNA content did not. Because the model showed that dry matter loss was not related to amount of fungal DNA, we show here indirectly that fungal decay cannot be the only or main cause of dry matter loss in this study. The dry matter loss in our study was most probably due to physical loss: needle and branch fall-off. Filbakk et al. (2011) documented the total undifferentiated dry matter loss in our material and we indirectly quantified the contribution of fungi.

3.3. Practical implications

Both drying (including fall off needles and twigs) and microbial activity will result in dry matter loss in logging residues. In Filbakk et al. (2011) about 20% total dry matter loss was measured for bundled residues. Although 20 % is a substantial loss, we show indirectly that the amount of fungal DNA detected could not cause this loss alone. Thus, storage of forest residues in piles, either bundled or loose, most probably will not be impaired by a substantial dry matter loss due to fungal decay. The substantial loss was most probably caused by the process of transpiration drying, causing the dry foliage and small twigs to fall off. The needle and twig fall off is considered beneficial because 1) its presence lowers the final combustion efficiency and 2) the nutrients contained in this material remain at the site as opposed to nutrient depletion by its complete removal.

We show that moisture is a key factor for fungal growth in piles. During storage time of maximum 460 days the moisture content in logging residues gradually decreased together with fungal DNA content. Hence, we show that storage of logging residues for maximum 460 days does not promote the fungal growth enough to significantly impair its quality.

Because we found higher values of fungal DNA in loose residues compared to bundles, the practice of bundling does not seem to promote more fungal growth than leaving the residues in loose piles. Neither did pre-storing of residues during winter seem to make a difference to fungal DNA content.

Our data suggest that fungal growth may be temporarily stimulated when the material has the optimal water content, such as in periods of increased precipitation. This supports the practice of covering the piles during storage.

Thus the harvesting and storage of logging residues in forests during prolonged periods does not pose a danger of the material being decomposed substantially by the fungi.

4. Conclusions

- We show that qPCR assay to quantify the fungal DNA is a sensitive method, capable of providing indirectly a measure of biodegradation in logging residues.
- Because the fungal DNA, indicating the presence of potentially decomposing basidiomycetes, decreases at the end of the storage period together with moisture content, there seems to be a small danger of fungi significantly contributing to dry matter loss of logging residues stored for less than 460 days in given conditions.
- Scots pine residues had more fungal DNA than Norway spruce residues.
- Loose piles had generally more fungal DNA than bundled ones.

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