

The Effects of Wood Storage on the Chemical Composition and Indigenous Microflora of *Eucalyptus* species Used in the Pulping Industry

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Lipophilic extractives naturally occurring in wood tend to coalesce during pulping to form pitch deposits, which have particularly undesirable effects on the pulping process and quality of pulp produced. A chemical characterization of different eucalypt species [*Eucalyptus nitens*, *E. grandis*, and *E. dunnii* (of different site qualities)] wood and generated pulp was performed. This study aimed at determining the effects of wood storage at -20 °C (for 6 months), by examining their chemical composition and indigenous microflora. Fatty acids were the main lipophilic compounds among *E. dunnii* (SQ3 and SQ4) and *E. grandis* wood extractives. The wood of *E. nitens* posed the least risk for pitch deposit formation, making it the most suitable *Eucalyptus* species for pulping. Storage of wood chips at -20 °C had a similar effect as the traditional method of seasoning (storage of wood outdoors prior to pulping) used for the reduction of lipophilic extractives. A 25 to 44% reduction of total extractives was observed in the raw material after storage. Variations in bacterial and fungal communities were observed after storage, and should be taken into consideration when conducting lab scale trials. If storage of wood chips is necessary for lab testing, it should be retained for a maximum of 3 months at -20 °C.

Keywords: Lipophilic extractives; *Eucalyptus*; Storage; Microflora

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INTRODUCTION

Eucalyptus species are the most extensively planted hardwood species in the world and are primarily used in the pulp and paper industry (Sappi 2012). Extractives make up the minor fraction of hardwoods but have the greatest potential to cause problems during pulping (Leskinen *et al.* 2015). The extractive content of wood is highly variable amongst species (Moodley 2011; Yang and Jaakkola 2011); therefore careful selection of *Eucalyptus* species for pulping is required and currently not taken into consideration in commercial pulping operations (Swain and Gardner 2003). The lipophilic fraction of wood extractives is responsible for the formation of pitch deposits during pulp production (Sjöström 1993; Kontkanen *et al.* 2004; Wang and Jiang 2006), resulting in poor pulp quality and gumming up of machinery (Hillis and Sumimoto 1989; Patrick 2004; Sarja 2007). Traditionally, pitch deposits are reduced by seasoning logs and wood chips (outdoor

storage of wood prior to processing) (Allen *et al.* 1991; Back and Allen 2000). Storage reduces the extractive content by hydrolytic or oxidative conversion by plant enzymes and the activity of indigenous microflora. The reduction of extractive content occurs much faster in woodchips rather than log form, as the oxidation processes proceed more easily and rapidly (Gutiérrez *et al.* 1998; Burnes *et al.* 2000). Temperature is also known to have an effect on the degradation of lipophilic compounds (Silvério *et al.* 2008). It has been reported that during winter the hydrolysis of wood esters, which forms part of the pitch, is drastically reduced compared to the summer months (Almeida and Silva 2001). In industry, seasoning wood at ambient temperatures usually significantly reduces the lipophilic content in the wood. However, care has to be taken, as prolonged seasoning could lead to uncontrolled action of microorganisms and insects (Bajpai 1999; Gutiérrez *et al.* 2001). The activity of such organisms could lead to cellulose and hemicellulose loss in the wood, ultimately resulting in poor pulp quality (Burnes 2000). Similarly, care has to be taken when storing wood for scientific investigations. Under laboratory conditions, wood chips are usually stored at -20 °C to prohibit any natural degradation of the lipophilic extractives, contamination by foreign organisms and to preserve the original state of the wood (Venn 1980; Petäistö 2006).

The lipophilic extractives of *E. globulus* and certain hybrids have been extensively characterized (Gutiérrez *et al.* 1999; Gutiérrez *et al.* 2001; Freire *et al.* 2002, 2006; Prinsen *et al.* 2012); however, there is a lack of information on extractives from *E. grandis*, *E. urograndis* (Freire *et al.* 2006), *E. urophylla*, *E. camaldulensis* (Silvério *et al.* 2007), *E. nitens*, and *E. dunnii* (Rencoret *et al.* 2007). In addition, limited information on the effect of site quality (soil quality and environmental conditions at the plantation site) on extractive content of *Eucalyptus* is available. Incorporating knowledge on the lipophilic content of different *Eucalyptus* species when selecting wood for pulping will go a long way towards reducing the overall lipophilic content of pulp, subsequently reducing the risks of pitch formation. In the present study, the chemical composition of the wood and pulp of four *Eucalyptus* species, *E. grandis*, *E. nitens*, *E. dunnii* site quality 3 (SQ3) and *E. dunnii* SQ4 were evaluated. Furthermore, the effects of storage at -20 °C on the chemical composition of different *Eucalyptus* spp. and their indigenous microflora were investigated.

EXPERIMENTAL

Samples

E. grandis, *E. nitens*, *E. dunnii* (SQ3), and *E. dunnii* (SQ4) were obtained from a commercial pulping mill in Umkomaas located on the south coast of South Africa. Wood chips from individual wood species were collected as they were being chipped in the wood yard. Samples were transported back to the laboratory and stored at -20 °C. Chip samples of the different wood species were milled using the Brabender® Wiley-mill to coarse sawdust. The sawdust was then passed through a 0.40 mm (40 mesh) screen and used for chemical analyses (TAPPI 2012). The following analyses were conducted on the milled wood chips before and after storage at -20 °C for 6 months. Time taken to mill and analyze the wood chips was up to 5 days. Wood chips were also pulped to determine any changes in the quality of the pulp.

Hot Water Extraction

Hot water (water soluble) extractives (waxes, fats, some resins, photosterols, and non-volatile hydrocarbons, low molecular weight carbohydrates, salts, and other water soluble substances) were evaluated by weighing out five grams of sawdust (moisture content of original sample recorded) in a 400 mL conical flask and slowly adding 100 mL of hot deionised water. The contents of the conical flask were well stirred to prevent the sawdust from floating. The conical flask was placed on a pre-heated hot plate and left to stand for 3 h. The initial level of the contents was noted and kept constant by the addition of hot deionised water. A pre-weighed number 3 crucible (Pyrex) and a vacuum pump were used to filter the sawdust which was washed with 200 mL of hot deionised water and dried at 60 °C overnight (TAPPI Test Method T204 om-88). Experiments were performed in triplicate.

Solvent Extraction

The Soxhlet extraction method was used to evaluate the solvent (water insoluble) extractives in the wood samples. Four grams of hot water extracted sawdust (moisture content of wet sawdust recorded) were weighed into an extraction thimble, which was placed in a Soxhlet apparatus that was attached to a pre-weighed 500 mL round bottomed flask containing 300 mL toluene-ethanol (2:1). The heating mantle was adjusted such that the solvent cycled through the system six times over 4 h. The extractives were rotor evaporated to dryness, cooled in desiccator and weighed (TAPPI Test Method T204 om-88). Experiments performed in triplicate.

Chemical Analyses

The other chemical characteristics of the wood chips were evaluated by conducting extractive analyses such as near infra-red reflectance analyser (NIRA) for the rapid quantification of wood chemical components and high performance liquid chromatography (HPLC) for quantification of cellulose and hemicelluloses (glucose, mannose, arabinose, xylose, rhamnose, and galactose) (TAPPI Test Methods 1996-1997; Wallis *et al.* 1996; Wright and Wallis 1996).

Pyrolysis GC-MS analysis

The lipophilic extractives present in the samples were ascertained by Py-GC/MS analysis. A multi-shot pyrolyzer, EGA/PY-3030 D, (Frontier Lab, Japan) attached to an ultra-alloy capillary column (30 m x 0.25 mm, 0.25 μ m) was used for analysis. The samples were pyrolysed at 550 °C for 20 seconds and the interface temperature was set at 350 °C. The chromatographic separation of the volatile components released by pyrolysis was performed using an ultra-alloy column. The injection temperature was set to 280 °C and the column flow rate was set to 1.0 mL/min with helium as the carrier gas. The temperature was programmed as follows: 50 °C for 2 min; rate 3 °C/min up to 200 °C and held for a further 4 min. The ion source and interface temperatures were set to 200 °C and 300°C, respectively. The scan range used for the mass selective detector was 40 to 650 m/z. Pyrolysis products were identified by comparing their mass spectra with the mass spectrum NIST library attached to instrument (modified from Sithole and Watanabe 2013).

Pulping

Pulping was performed in duplicate according to the acid bi-sulphite process currently implemented in Dissolving Wood Pulp (DWP) mills. Pulp quality was determined by assessing viscosity (TAPPI T203 om-94), final pulp yield, kappa number, acid insoluble lignin (TAPPI T222 om-88), alpha cellulose, S8, S10 (TAPPI T235-OM60) and hemicelluloses (Forestry and Forest Products Research Centre, CSIR, Personal Communication 2014).

DNA Isolation and Polymerase Chain Reaction (PCR)

DNA was extracted from 0.2 g milled chips using a kit as per manufacturer's specifications (Soil DNA Extraction Kit, Zymo Research). Ribosomal genes were amplified from microbial genomic DNA using 16S and ITS2 region of 18S rRNA genes with the universal primer sets: 63F/1387R (Marchesi *et al.* 1998) and ITS5F/ITS4R (White *et al.* 1990), respectively. Each amplification reaction (50 μ L) contained 1.25 mM MgCl₂, 0.125 μ M forward and reverse primers, 0.2 mM deoxynucleoside triphosphate (dNTPs), 0.25 U SuperTherm Taq DNA polymerase (Southern Cross Biotech), and 20 to 200 ng of template DNA. PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems). For amplification of 16S rRNA, PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation, annealing and extension at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 5 min, whereas 18S rRNA amplification conditions were: initial denaturation at 95 °C for 2 min, 25 cycles of 95 °C for 30 sec, 53 °C for 45 sec, 72 °C for 1 min and a final extension at 72 °C for 8 min. The amplicons were analyzed by electrophoresis on 1% agarose (SeaKem) gels in 1 \times TAE running buffer at 90 V for 45 min. After electrophoresis, the gels were stained in 0.5 μ g/mL ethidium bromide and visualized using the Chemi-Genius 2 BioImaging System (Syngene). Upon confirmation of 16S and 18S amplicons, products were purified using a GeneJET™ PCR purification kit (Fermentas) and re-amplified in a touchdown thermal profile program using nested PCR and primers with GC clamps. PCR for 16S rRNA genes were initially performed using two primer sets: 338F-GC with a GC-clamp: 5'-CGCCCGCCGCGCGCGGGCGGGCGGGGCGGGGGCACGGGGGG-3' and 518R 211 (237-bp fragment) (Handschr *et al.* 2005); 933F-GC with a GC-clamp: 5'-CGCCCGCCGCGCGCGGGCGGGCGGGGCGGGGGCACGGGGGG-3' and 1387R (500-bp fragment) (Ji *et al.* 2004). The composition of the reaction mixtures was the same as that used for the first PCR. For amplification of 16S rRNA, PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation annealing and extension at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 5 min, whereas 18S rRNA amplification conditions were: initial denaturation at 95 °C for 2 min, 25 cycles of 95 °C for 30 sec, 53 °C for 45 sec, 72 °C for 1 min and a final extension at 72 °C for 8 min (Schabereiter-Gurtner *et al.* 2001).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using the D-Code Universal Mutation Detection System (BioRad), and an optimized method (Ramnath *et al.* 2013) modified from Muyzer and Smalla (1998). PCR samples were loaded onto vertical perpendicular polyacrylamide gels (6% acrylamide:bisacrylamide [37.5:1]) in 1 \times TAE buffer prepared using 30 and 60% denaturant (100% denaturant corresponds to 7 M urea and 40% formamide). Gradients of

30% and 60% were optimal for bacterial amplicons (~237 bp) and 25% and 50% for fungal amplicons (~316 bp). A pre-run was performed at a constant voltage of 150 V at 60 °C for 30 min, following which, DGGE profiles were generated at a constant voltage of 60 V in 1× TAE buffer at 60 °C for 16 h. After electrophoresis, gels were stained in 0.5 µg/mL ethidium bromide for 60 min, destained in the same volume of 1× TAE buffer for 30 min and visualized using the Chemi-Genius 2 BioImaging System (Syngene).

RESULTS AND DISCUSSION

Hot water extraction is known to be an efficient technique for the removal of hemicellulose from wood chips prior to pulping in the production of dissolving pulp (Li *et al.* 2010). A comparison of the samples before and after storage at -20 °C revealed a general decrease in hot water and solvent extractives (Fig. 1).

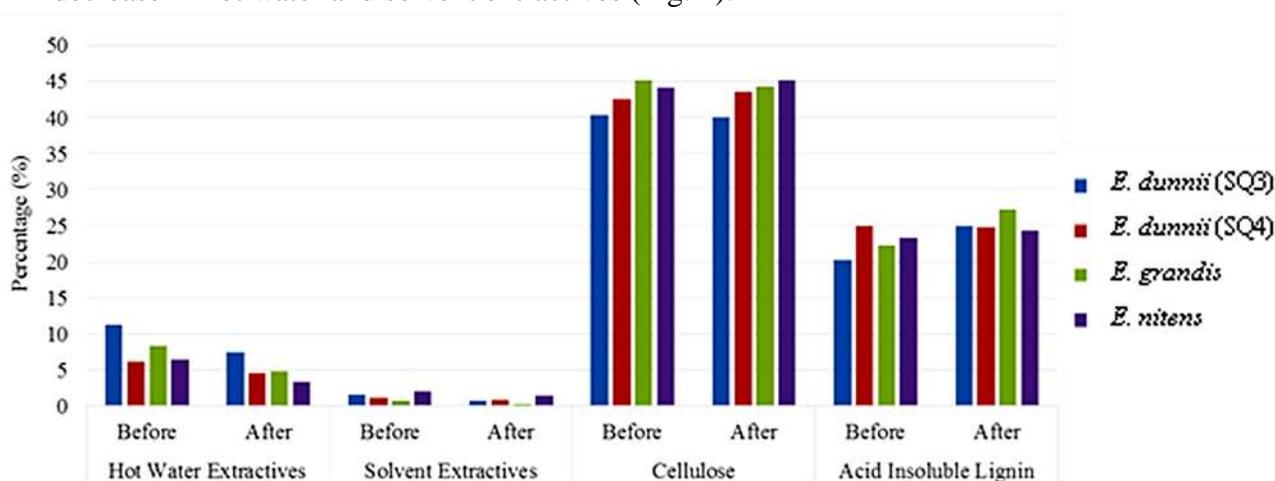


Fig. 1. Wet chemistry of wood chips of different *Eucalyptus* spp. before and after storage at -20°C.

The highest hot water extractive content was observed for *E. dunnii* SQ3 (11.2%). The extractive contents are known to vary from tree to tree and considerably from species to species (Hillis 2014). *E. dunnii* SQ3 exhibited higher hot water extractive (11.2%) and solvent extractive (2%) content compared to SQ4 (6% and 1.7%, respectively), demonstrating the variation in chemical characteristics amongst *Eucalyptus* wood of the same species but from different site qualities. This is supported by Kilulya *et al.* (2014) who also observed that variations in lipophilic content are influenced by tree species and site qualities. Furthermore, their study validates our finding of higher amounts of total lipophilic extractives in *E. dunnii* compared to *E. grandis*. Wood chips with a hot-water soluble content exceeding 7 to 8% (as seen with *E. dunnii* and *E. grandis* in this study) result in obstruction of delignification due to competition of extractives with lignin for active cooking chemicals during pulping (Back and Allen, 2000). Consequently, higher amounts of active alkali are required which increases the cost and waste generated (Cohen and Mackney 1951). Extractives may cause liquor decomposition, also resulting in higher chemical requirements per ton of pulp produced (Hillis 2014).

High performance liquid chromatography (HPLC) was used for quantification of hemicelluloses (glucose, mannose, arabinose, xylose, rhamnose, and galactose) in the wood chips before and after storage. An increase in xylose content after storage for all *Eucalyptus* species was observed. This may be attributed to the natural degradation of glucuronoxylan during storage (Table 1). Pentosans abundant in hardwoods are mostly glucuronoxylan. The bonds between xylose units in glucuronoxylan are simple to hydrolyze (Testova *et al.* 2009), highlighting the potential for natural degradation during storage. Galactose and arabinose levels decreased for all *Eucalyptus* spp. after storage with the greatest decrease observed in *E. nitens*. This decrease during storage may be attributed to microbial degradation. Previous studies have shown that during the initial stages of fungal degradation, the carbohydrates most modified were sugars derived from the primary walls and middle lamella (galactose and arabinose). These are often recognized as the building blocks of hemicelluloses and/or arabinogalactan proteins in the primary wall (Skyba *et al.* 2013).

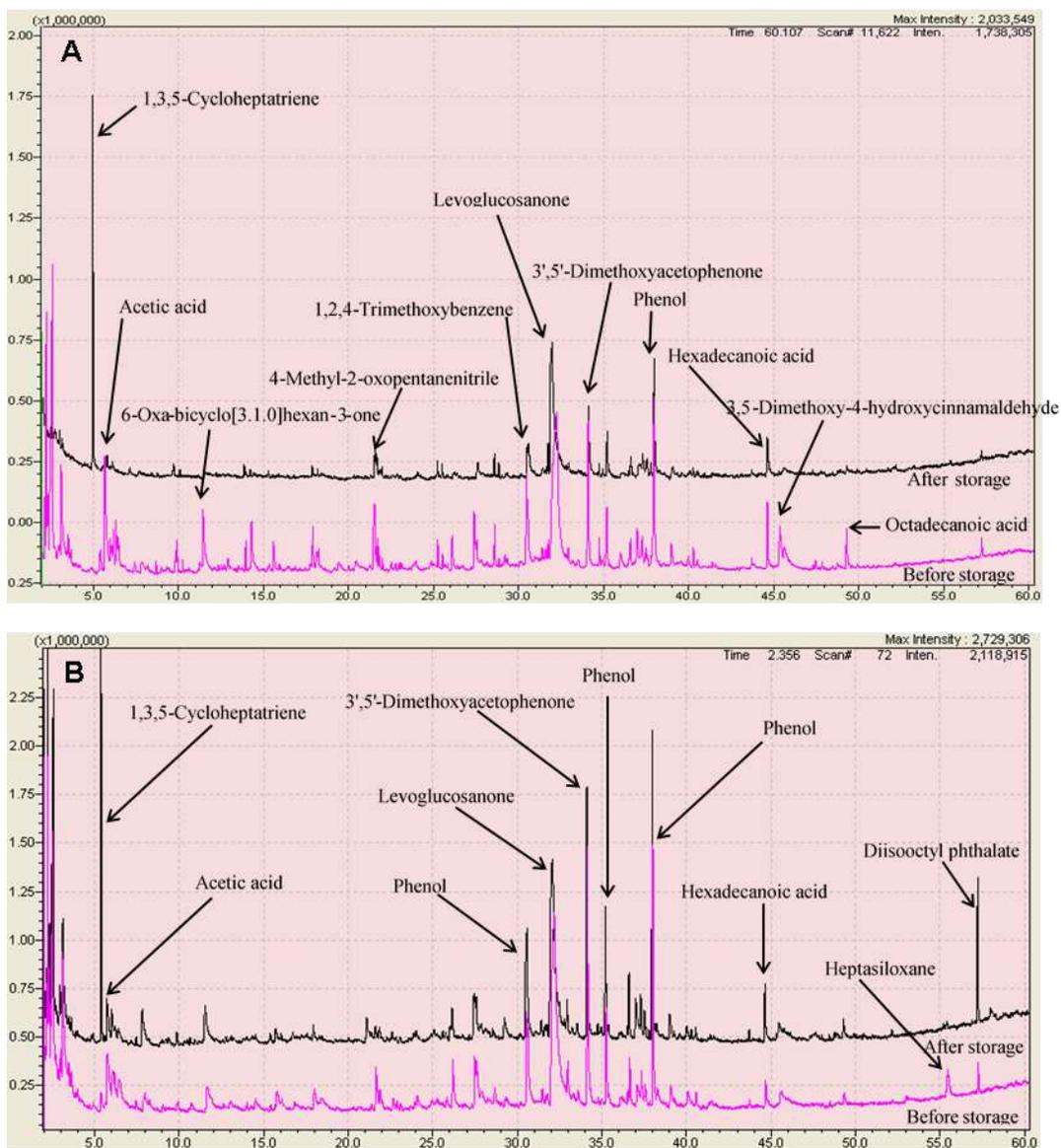
The mannose content of the wood material decreased by 24%, 21%, and 9% for *E. dunnii* (SQ3), *E. grandis*, and *E. nitens*, respectively, after storage. This decrease in mannose content may be caused by the activity of hemicellulolytic enzymes produced by microorganisms indigenous to the wood, particularly with *E. dunnii* (SQ3) and *E. grandis*. Wood treated with specific enzymes enable the extraction of high molecular weight materials, whilst non-specific enzymes were found to degrade hemicelluloses to a large extent (Azhar 2015). Thus, the increase in sugars observed in this study may be attributed to non-specific hemicellulolytic activity of indigenous microflora found in the wood material.

Table 1. Carbohydrate Content of Wood Chips Before and After Storage at -20°C

	Arabinose %		Galactose %		Glucose %		Xylose %		Mannose %	
	Before	After	Before	After	Before	After	Before	After	Before	After
<i>E. dunnii</i> (SQ3)	0.41	0.32	1.43	1.15	49.40	48.09	9.05	10.68	1.31	1
<i>E. dunnii</i> (SQ4)	0.27	0.19	0.95	0.70	49.18	49.84	10.82	11.24	1.44	1.51
<i>E. grandis</i>	0.27	0.16	1.04	0.79	50.62	49.24	7.83	9.97	1.74	1.37
<i>E. nitens</i>	0.16	0.09	0.65	0.37	50.37	51.5	9.87	11.35	1.69	1.54

Py-GC-MS analyses provided insight into the lipophilic content of the samples. The py-GC-MS profiles of the different wood species in Fig. 2 show that the samples contained mainly lignin (3',5'-dimethoxyacetophenone) and lipophilic (octadecanoic acid and hexadecanoic acid) degradation products due to pyrolysis. Storage at -20 °C had a discernible effect on the lipophilic content of the wood. The hexadecanoic and octadecanoic fatty acids observed are an indication of polymerised lipids. Polymerisation is the cause of incomplete removal of lipids by extraction (Tao *et al.* 2010). Reductions in these fatty acids were observed for *E. dunnii* (SQ4), *E. nitens*, and *E. grandis*. Such reductions would contribute to a decrease in pitch deposit formation and would usually be considered as a positive effect. These results confirm that storage at -20 °C resulted in degradation of pitch components as with aspen wood chips reported by Allen *et al.* (1991). However, if the wood chips are being stored for future experimental use, any changes from the original state of the wood would not be considered favourable. *E. grandis* (Fig. 1D)

contained significant amounts of higher molecular weight lipophilic extractives (hexadecanoic and octadecanoic fatty acids), compared to the other wood species. These compounds were present in the other samples but at much lower levels, reflecting the inherently greater lipophilic content of *E. grandis*. Extractives were slightly lower than what has been reported for *Eucalyptus* species, which may indicate that samples were not as fresh and extractives may have transformed into higher molecular weight lipids (Kilulya *et al.* 2014). The phthalate and siloxane observed in *E. dunnii* (SQ3) are contaminants and are not part of the wood. Traces of phthalates and siloxane are frequently found in most industrial samples, and inevitably form part of the surface composition (Fardim and Durán 2003).



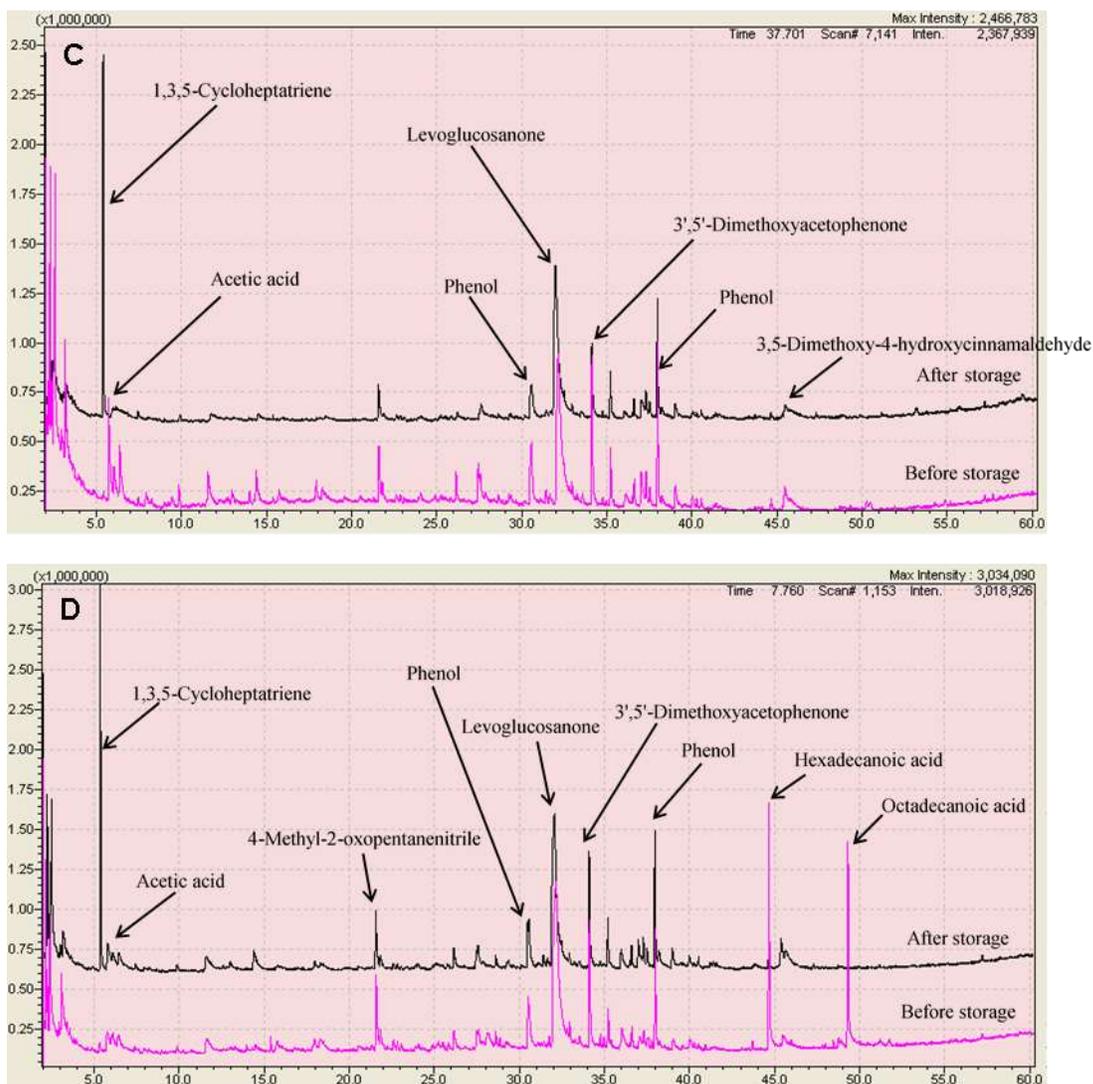


Fig. 2. Lipophilic profiles of different *Eucalyptus* species, before and after storage at -20 °C. (A) *E. dunnii*, (B) *E. dunnii* (FFP), (C) *E. nitens*, (D) *E. grandis*

The pulp produced from the wood chips was evaluated before and after storage, by measuring basic pulp chemistry: kappa number, viscosity, pulp yield, alpha cellulose, S10 and S18, and copper number. The kappa number provides an estimate of the amount of chemicals required during bleaching of wood pulp to achieve a pulp with a specified degree of whiteness. Therefore, the amount of bleach required is correlated to the lignin content of the pulp. Kappa number is used to determine the effectiveness of the lignin-extraction phase of the pulping process. It is approximately proportional to the residual lignin in the pulp (Costa and Colodette 2007). All *Eucalyptus* species demonstrated a reduction in the total lignin content of the pulp (Table 3). Greater removal of lignin may have been possible due to the opening up of the wood structure during storage by the enzymes produced by

the indigenous microflora present. The pulp produced after storage had a lower kappa number than the starting material, but with a relatively low pulp viscosity (Table 2). Since the hemicelluloses are able to shield the cellulose chain from alkaline hydrolysis (Lindström and Teder 1995), the low pulp viscosities after storage are possibly due to removal of protective hemicelluloses (Azhar 2015). Pulp viscosity (degree of polymerization of cellulose fibers) reveals the relative consistency of the cellulose fibers after pulping. The pulp viscosity varied between the different *Eucalyptus* species from 28.01 to 131.7 cP. A general decrease in the viscosity was observed for all samples after 6 months of storage. Viscosity values for *E. dunnii* (SQ3) were very low and may be explained by the degradation of cellulose fibers by cellulase-producing microorganisms previously isolated from this particular wood species (Ramnath *et al.* 2013). A considerable decrease in viscosity of 41% and 66% in *E. grandis* and *E. nitens* pulp, respectively, was observed after storage. This may be attributed to endoglucanase activity, which causes random cleavage of cellulose chains leading to lower degrees of polymerization and therefore lower viscosity. The retention of long cellulose fibers is essential for producing dissolving pulp (high grade cellulose pulp). Cellulase activity results in cellulose degradation; thus the negative effect of cellulases on pulp needs to be eliminated or minimized.

After storage, glucose levels varied in the range 80.85 to 86.19% and xylose from 5.27% to 6.44% (Table 3). This provides an approximate indication of the cellulose to hemicellulose ratio amongst the different species (Neiva *et al.* 2014). *E. dunnii* (SQ3) and *E. dunnii* (SQ4) displayed the lowest and highest ratio of glucose/xylose, respectively. These results support the notion that site quality does have an effect on the chemical composition of the wood material within individual species. The hemicellulose content in *E. dunnii* (SQ4) pulp was higher than the other *Eucalyptus* species examined. A general increase in xylose content (Table 3) was observed for all *Eucalyptus* pulp samples. Fišerová *et al.* (2013) found that xylose content of hydrolyzed beech and oak wood samples increased with increasing wood weight loss. Hence the increase in xylose observed may be attributed to wood weight loss during storage, which may be caused by wood-decaying fungi that became activated once thawed. Wood-decaying fungi have acquired a range of hydrolytic enzymes including cellulases, hemicellulases, and oxidative enzymes for the breakdown of lignocellulose into free monomers such as xylose, mannose, glucose, galactose, arabinose and rhamnose (Álvarez *et al.* 2016). In another study, greater xylose levels were observed when *Eucalyptus* and rice straw materials were treated with a culture supernatant of *Trichoderma reesei* than with traditional steam explosion as a pretreatment step (Álvarez *et al.* 2016). This emphasizes the xylan-hydrolyzing potential of *T. reesei* to release xylose oligomers and other soluble sugars (Jørgensen *et al.* 2007), and may explain the higher xylose concentrations documented in this study. In addition, xylanases may assist in the pulp bleaching process, by removing xylan which allows for easier bleaching of the cellulose fibers, therefore consuming less bleach and energy (Garg *et al.* 2011). This partial degradation of xylan could also assist in the degradation of lignin by other microorganisms present, which is essential as lignin also impedes pulp bleaching (Subramaniyan and Prema 2002).

Table 2. Characteristics of the Pulp Produced from Wood Chips Before and After Storage

	SPY		Viscosity (cP)		K-number		S10		S18		S10-S18		Alpha cellulose		Copper number	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
<i>E. dunnii</i> (SQ3)	49.68	48.65	28.01	27.98	4.06	3.76	9.77	9.78	7.08	6.93	2.69	2.85	91.56	91.57	3.15	3.15
<i>E. dunnii</i> (SQ4)	49.96	48.02	90.26	81.56	4.39	3.34	10.04	10.54	7.95	7.23	2.09	3.31	91.29	90.08	2.93	3.04
<i>E. grandis</i>	49.46	47.54	74.04	43.73	4.58	3.85	11.04	9.85	8.20	6.29	2.84	3.56	90.38	91.67	3.06	2.61
<i>E. nitens</i>	50.78	44.77	131.7	44.77	5.04	2.31	10.06	11.36	7.36	6.09	2.70	5.27	91.01	90.81	2.47	2.53

SPY – screened pulp yield

Table 3. Quantification of Lignin and Hemicelluloses of the Pulp Produced from Wood Chips Before and After Storage

	Glucose (%)		Xylose (%)		Mannose (%)		Klason lignin (%)		Acid insoluble lignin (%)		Total lignin (%)	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
<i>E. dunnii</i> SQ3	90,76	84.69	2,82	5.27	1,49	0.83	2,24	2.02	1,69	1.61	3.93	3.63
<i>E. dunnii</i> SQ4	91.07	86.19	5.37	6.44	1.15	0.77	2.63	2.14	1.82	1.91	4.46	4.05
<i>E. grandis</i>	90,35	81.02	3,63	5.72	2,55	1.47	1,96	1.68	1.78	1.28	4.91	2.96
<i>E. nitens</i>	90,94	80.85	4,97	5.93	1,35	0.95	1,25	1.04	1,82	2.02	4.59	3.06

Prior to storage, pulping of *E. nitens* wood resulted in the highest pulp yield of 50.8%; however, after storage the yield was reduced to 44.8%, the lowest level amongst the *Eucalyptus* spp. examined. After storage *E. dunnii* (SQ3) generated the highest pulp yield of 48.6%. Considering the pulping characteristics of the different species without the effect of storage, *E. nitens* would be the most suitable for pulping as it generated the greatest pulp yield (50.8%) and highest pulp viscosity (131.7%). Integrating the effects of storage and *E. dunnii* (SQ4) would be the most suitable for pulping as minimal effects on pulp yield and viscosity were observed for this species.

Following successful DNA isolation and PCR, DGGE analysis of the bacterial communities present in the wood revealed some interesting findings. There was a significant increase in the diversity and abundance of bacteria present in the wood samples after 6 months of storage (Fig. 3). The microbial community of harvested crops is known to transform during storage, and is particularly dependent on the water content of the samples (Barry Kay *et al.* 2009). Identities of the microorganisms were inferred using a molecular weight marker of known microbial species previously isolated from *Eucalyptus* wood chips. *Bacillus thuringiensis* and *B. cereus* appeared to be prevalent in all of the samples after storage, except for *E. dunnii* (SQ4). In fact, the bacterial community detected in *E. dunnii* (SQ4) post storage was minimal compared to the other *Eucalyptus* species. Following storage of *E. grandis*, DGGE analysis revealed the presence of *Bacillus thuringiensis*, *Klebsiella* sp., *Pantoea ananatis*, *Micrococcus luteus*, and *Inquilineus limosus*, and the subsequent loss of *Prauserella* and *Saccharomonospora* spp. *E. nitens* on the other hand, showed loss of the *Inquilineus limosus* population and increase in intensity of *Lecleria* sp., *Saccharomonospora* sp., and *B. cereus* populations.

Fungal diversity amongst the different *Eucalyptus* species was evident. For all *Eucalyptus* species, various fungal species observed prior to storage, increased in abundance after storage. This was observed for the following fungal species; *Phialophora alba* (*E. dunnii* SQ3 and *E. grandis*), *Brachyalarca straminea*, *Loddermayces elongisporus* (*E. dunnii* SQ4), *Aspergillus fumigatus* (*E. grandis*), and *Basidiomycota* sp. (*E. nitens*). In addition, fungal species such as *Paecilomyces variotii*, *Curvularia* sp., and *Pichia scolytii* were discovered only after storage in *E. dunnii* (SQ3), *E. dunnii* (SQ4), and *E. grandis*, respectively. This may seem anomalous, as at temperatures of -10 to -12°C, microbial growth is considered to be minimal. However, some microorganisms remain inactivated during storage and then continue growing once thawed, leading to microbial degradation of the thawed product (Hui and Sherkat 2005). Freezer storage of material may eliminate the risk of microbial activity during the storage period, but wood material is still at risk during the cooling-down and thawing processes (Hansen 1990). Petäistö (2006) found that the growth of grey mold, such as *Botrytis cinerea* commonly found in soil, on Norway spruce progressed at the beginning and/or thawing phase of cold storage at -3°C. Temperatures above zero before and after the cold-storage phase may intensify the threat of grey mold damage although the optimum temperature for *B. cinerea* is approximately 20 °C (Petäistö 2006).

Low concentrations of extractives in wood have previously been associated with low resistance to decay, as extractives may function as a fungicide (Haupt *et al.* 2003; Latorraca *et al.* 2011; Severo *et al.* 2016). The increase in microbial populations and diversity may have been facilitated by the lower extractives content observed in the tested samples.

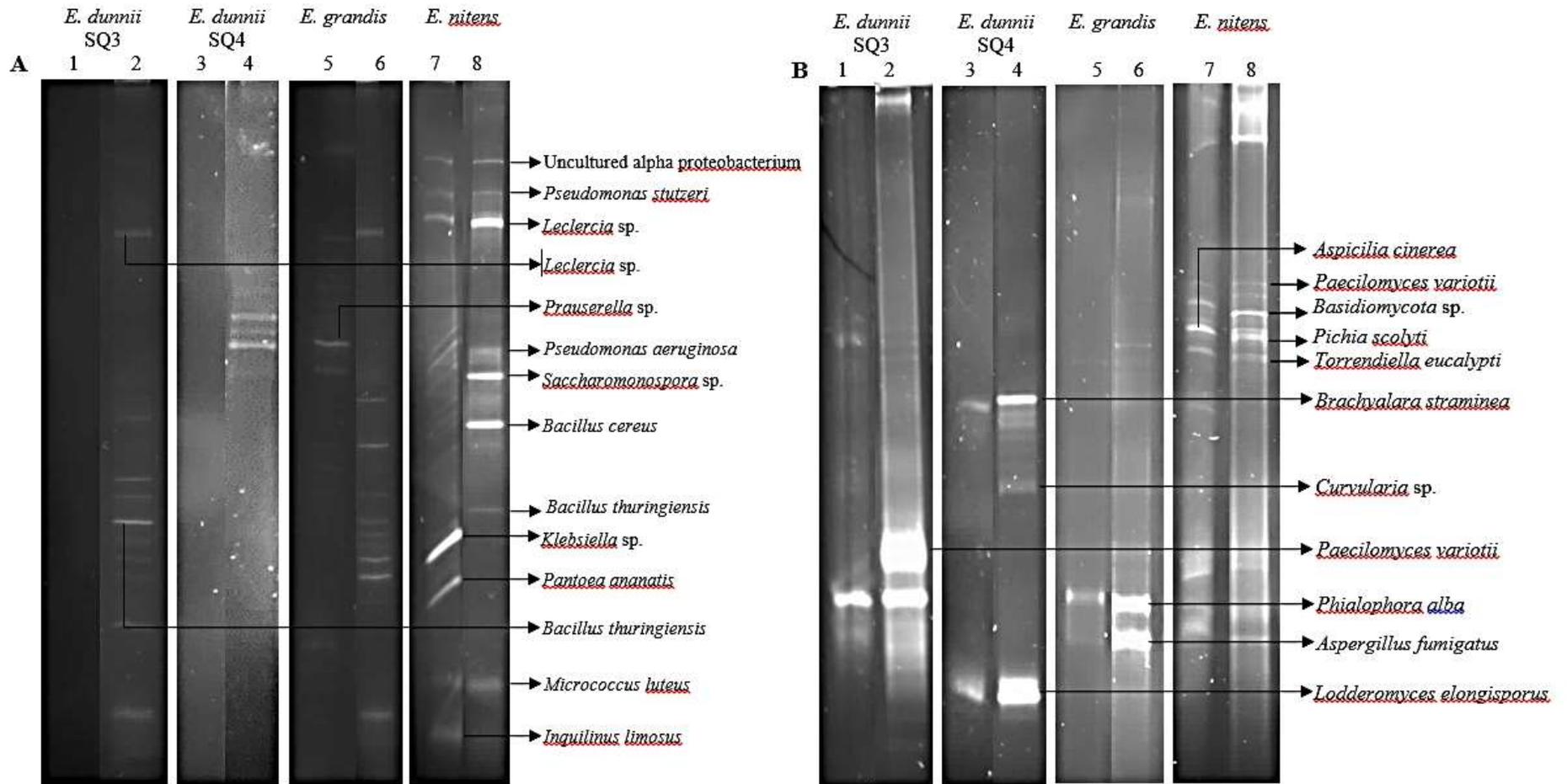


Fig. 2. DGGE profiles of bacterial (A) and fungal (B) communities present in different *Eucalyptus* species before and after storage at -20°C. Lanes: 1- time zero *E. dunnii* (SQ3); 2- after storage *E. dunnii* (SQ3); 3- time zero *E. dunnii* (SQ4); 4- after storage *E. dunnii* (SQ4); 5- time zero *E. grandis*; 6- after storage *E. grandis*; 7- time zero *E. nitens*; 8- after storage *E. nitens*

The microorganisms identified here have been described in other studies on hardwood chips, including *Paecilomyces* sp., *Phialophora* sp., *Bacillus* sp., *Pseudomonas* sp., and *Micrococcus* sp. (Adair *et al.* 2002; Rajala *et al.* 2010; Kropacz and Fojutowski 2014; Zhang *et al.* 2014; Szulc *et al.* 2017). The microorganisms detected in the wood chips of different *Eucalyptus* species have the potential to produce cellulolytic, hemicellulolytic, and xylanolytic enzymes (Schmidt 2006; Seo *et al.* 2013; Nandimath *et al.* 2016), which would affect the chemical composition of the wood material. *E. dunnii* (SQ4) exhibited the lowest diversity of indigenous microorganisms, which supports the idea of minimal degradation of cellulose fibers as evidenced by the high pulp yield and viscosity levels obtained for this species, making it the most suitable species for pulping.

Some of the bands on the gels remain unidentified, therefore the microbial diversity may be underrepresented. However, the principle of DGGE that one band represents one genus/species is not always correct as a single point mutation occasionally results in two bands (Miller *et al.* 1999; Muyzer and Smalla 1998; Adil 2015). Therefore, there is the possibility of multiple bands for a single species due to single base pair mutations in their DNA.

Numerous changes in the chemical and biological nature of the wood were observed after six months of storage at -20 °C. This is unfavorable, particularly if the wood is to be used for experimental purposes. Previous studies have shown that storage of wood chips at -20 °C storage retards seasoning but does not stop it altogether (Allen *et al.* 1991; Dunlop-Jones *et al.* 1991; Back and Allen, 2000). The authors therefore recommend that wood material be stored for a maximum of three months under laboratory conditions. This recommendation is based on information from available literature (Allen *et al.* 1991; Back and Allen 2000).

CONCLUSIONS

1. Storage of wood chip samples at -20 °C influenced the chemical nature of *Eucalyptus* wood, particularly the lipophilic extractives.
2. This effect was similar to that seen with the traditional method of seasoning used for the reduction of lipophilic extractives. This is contrary to reports that storage of wood chips at -20 °C retards seasoning.
3. Changes in bacterial and fungal communities were observed after storage, which should be taken into consideration when conducting lab scale trials.
4. It is therefore recommended that if storage is necessary under laboratory conditions, it should be retained for a maximum of 3 months at -20 °C.

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