SEASONAL VARIATION OF MICROFLORA AND THEIR EFFECTS ON THE QUALITY OF WOOD CHIPS INTENDED FOR PULPING

BY

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Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus).

As the candidate's supervisor, I have approved this dissertation for submission.

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PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences; University of KwaZulu-Natal (Westville Campus), Durban, South Africa from March 2010 to December 2012, under the supervision of Dr. R. Govinden and the co-supervision of Dr. T. Bush.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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Publication 1

Method Optimization for DGGE Analysis of Microflora from Industrial Wood Chips Intended for Pulping

Online International Journal of Microbiology Research **(*in press*)**

Publication 2

DGGE Analysis of Bacteria and Fungi in Commercial Wood Chip Piles

Journal of Applied Microbiology and Biotechnology **(submitted to journal)**

Publication 3

Strategic Combination of *Eucalyptus* species for the Potential Management of Pulp Quality

Journal of Applied Microbiology **(submitted to journal)**

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ABSTRACT

Eucalyptus, pine and wattle are the predominant exotic wood species used in the production of dissolving pulp in South Africa. On entering the mill, wood is chipped and stored in outdoor piles where it becomes vulnerable to microbial degradation and spontaneous combustion. Major losses of stored chips are due to high temperatures and combustion caused by heat energy released by microbial fermentation. Changes in the chemistry of the wood chips caused by the metabolic activity of indigenous microflora combined with the inherent chemical characteristics of each wood species could have a potential impact on final pulp quality and yield. Therefore the objective of this study was to analyse the microbial (bacteria and fungi) communities present in commercial wood chip piles and correlate this with changes in the chemistry of the wood in summer and winter.

The molecular fingerprinting technique of Denaturing Gradient Gel Electrophoresis (DGGE) was optimized for the detection of microbial diversity in commercial wood chips. Wood chips were collected from an industrial wood yard and milled to different specifications. A total of four primer sets with GC-clamps were tested in nested PCR for DGGE analysis. 16S and 18S rRNA genes were amplified using 338f-GC/518r; 933F-GC/1387R (bacteria) and NS26/518R-GC; EF4F/518R-GC (fungi), respectively. Several gel gradients were examined to determine optimal separation of bacterial (40/60%, 35/50%, 30/60%) and fungal (35/50%, 20/45%, 25/50%) PCR-DGGE products. Comparison of the DGGE profiles revealed greater diversity in the milled wood chips amplified using primer sets; 338F-GC/518R (16S) and NS26/518R-GC (18S) with gradients of 30/60% (16S) and 25/50% (18S). Once optimized, this standardized protocol was tested against five samples to assess its applicability to woodyard samples. 16S and 18S DGGE profiles were generated and amplicons excised from gels, re-amplified, sequenced and the microorganism from which the DNA originated was determined. In the second phase a cross-sectional study of wood chip piles from a commercial dissolving pulp mill was conducted with sample collected in summer and winter using the optimized PCR-DGGE technique. Microbial strains were identified after sequencing of 16S and 18S rRNA amplicons separated by DGGE. Chemical characteristics of the wood chips were evaluated by conducting extractive analyses using HPLC. Due to unpredictable combinations of different wood species in commercial wood chip piles, the third phase involved the investigation of individual *Eucalyptus* species. The microflora indigenous to the two *Eucalyptus* species (*E. dunnii* and *E. nitens*) and a combination of the

two were subjected to winter and summer simulations for one month during which samples were tested for wood chemistry properties, microflora and the final samples were used to generate dissolving pulp.

Using the PCR-DGGE method eighteen bacterial and twelve fungal species were identified from the five samples collected from the commercial wood chip pile, compared to the ten bacterial and nine fungal isolates which were identified using the culturing technique and standard 16S and 18S rRNA gene sequence analysis. Predominant genera in the optimization phase of this study were *Klebsiella* spp. (×3), *Bacillus* spp. (×2), *Pantoea* spp. (×2), *Pseudomonas* spp. (×2) and *Paecilomyces* spp. (×2). Application of the optimized DGGE technique to samples collected from the commercial pulping mill in summer and winter revealed variable profiles indicating a range of bacterial and fungal strains that varied in intensity in the areas and seasons sampled. Seventy nine (45 in summer and 34 in winter) and 29 (20 in summer and 9 in winter) distinct amplicons representing bacteria and fungi, respectively, were visualized. Predominant genera in summer were *Pantoea rodasii*, *Inquilinus limosus, Streptococcus* sp., *Klebsiella* spp., *Diversispora* sp., *Boletaceae* sp., *Scutellospora* sp., and *Ophiostoma bicolour*. In winter the prevailing genera were *Leuconostoc palmae*, *Streptococcus* sp., *Bacillus* spp., *Diversispora* sp., *Boletaceae* sp., and *Bullera* sp. Lower cellulose levels in summer correlated significantly with high microbial loads and the predominance of *Bacillus* spp., suggesting that in warm humid environments storage should not exceed 1-2 weeks. No correlations were determined between the decreased hot water levels in winter and microbial activity, however they were correlated to increased exposure of those samples to environmental factors. Chemistry data on the wood chips imparts the quality of the wood which only permitted projection of final pulp quality. This inadequacy was addressed in the third phase which included identification of microbial strains, originating from the individual *Eucalyptus* species, after sequencing of 16S and 18S rRNA amplicons separated by DGGE. Fungal and bacterial species were also isolated, cultured, identified and screened for lignocellulolytic enzyme activity. Ninety two and 88% of the fungi isolated were capable of producing cellulase and xylanase, respectively. Significant correlations exist between the microflora, seasons (greater diversity and loading in summer) and the chemical and physical properties of wood chips (lower cellulose and viscosity in summer) as well as *Eucalyptus* species (significantly higher cellulose and viscosity for the combination and *E. nitens*). Indigenous microflora of each wood species may be one of the contributing factors to poor/good pulp quality, as significant correlations

were made between enzyme production of microorganisms and wood chemistry which ultimately has an impact on the final pulp quality and yields. This investigation provides proof of concept that combining wood species with different deterioration rates results in an overall improvement in pulp quality and thus paves the way for a practical and applicable approach to managing quality of chips.

1.1 INTRODUCTION

Reduced paper consumption and increased application of high quality cellulose pulp has elevated production of dissolving pulp. Efforts to reduce pollution have initiated interest in green biotechnology whilst improving pulp quality using biopulping and biobleaching techniques (Savitha *et al*., 2009). In the textile and papermaking industries, pulp is derived from chemical and mechanical treatment of wood chips. On entering the mill, wood chips are stored in piles and become vulnerable to degradation by microbiological attack (Fuller, 1985; Lehtikangas, 2000) and spontaneous combustion (Li *et al*., 2006). The parenchyma cells in the wood chips continue to respire in an attempt to repair the tree; thus oxygen is consumed and heat released, which provides favorable growth conditions for bacteria and subsequently fungi which feed on wood extractives (Fuller, 1985). Bacteria and actinomycetes are common wood-inhabiting microorganisms and initial wood colonizers (Clausen, 1996). During the pulping process, contaminated or damaged wood chips may become overcooked leading to poor pulp quality. The first part of this review describes the nature of wood, and its use in the pulp and papermaking industry. The second part serves to describe various fungi and bacteria involved in the biodegradation of wood and the practical application of their enzymes in biopulping.

1.2 STRUCTION AND COMPOSITION OF WOOD

Wood is generally divided into two major groups: softwoods, i.e., gymnosperms (pine) and hardwoods, i.e., angiosperms (*Eucalyptus*) (Argyropoulos and Menachem, 1997). It is mainly composed of empty, elongate, spindle-shaped cells which are positioned parallel to each other down the trunk of the tree (Miller, 1999). Cells vary from 16 to 42 µm in diameter and from 870 to 4000 µm long (Winandy, 1994). Each of these cells has four cell wall layers (Fig. 1), which are Primary (P), S_1, S_2, and S_3 (Winandy, 1994). The nature of these fibre cells may alter the strength and shrinkage of the wood, including its grain pattern. Cellulose, hemicellulose and lignin form the main components of wood. The majority of the cell wall is made up of crystalline cellulose. Lignin is located primarily towards the outside of the cells and between the cells (Miller, 1999).

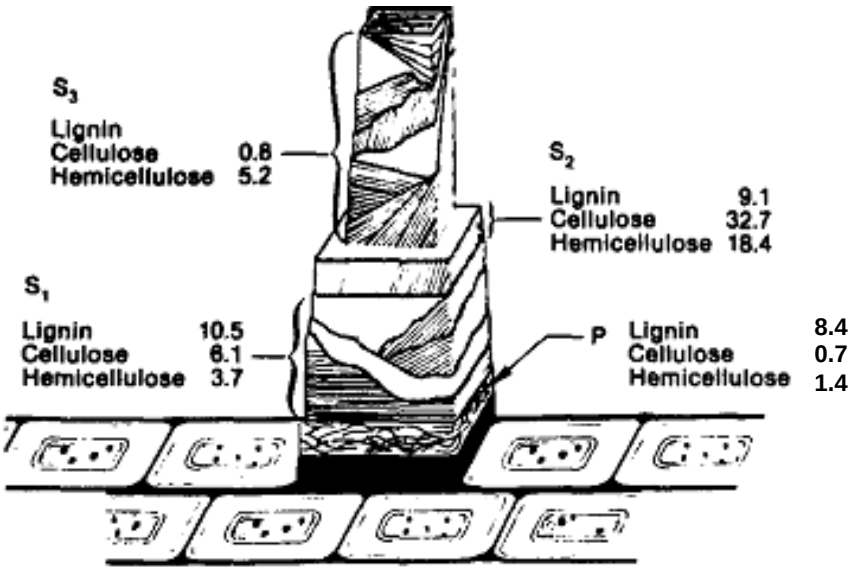

Figure 1: Microfibril orientation for each cell wall layer of Scots pine (See Table 1) with chemical composition as percentage of total weight. Cell wall layers are primary (P), S1, S2, and S3 (Winandy, 1994).

1.2.1 Cellulose

Cellulose is the main constituent of wood, and forms 40-50% in both hard and softwoods (Table 1) (Sjostrom, 1993). Cellulose is composed of several thousand D-glucose units linked by β-1,4-glycosidic bonds with a 10 000 degree of polymerization in native wood and 1 000 in bleached kraft pulps (McMurry, 2000; Klemn *et al*., 2004). In the plant cell walls it is grouped into microfibrils, which are grouped into fibrils and further grouped to form cellulose fibre. These fibres provide strength and rigidity to plants (Argyropoulos and Menachem, 1997; Campbell and Reece, 2002). In the pulp and paper industry the retention of cellulose is vital in generating strong pulp. The degradation of cellulose into glucose requires the synergistic action of three major cellulases: endoglucanases, exoglucanases and β-glucosidases (Lynd *et al*., 2002). Microorganisms have the ability to produce these enzymes singularly or as multi-protein complexes (Lynd *et al*., 2002).

Table 1: Chemical composition of some wood species (Sjostrom, 1993)

Constituent	**Scots Pine (*Pinus sylvestris*)**	**Spruce (*Picea glauca*)**	**Eucalyptus (*Eucalyptus camaldulensis*)**	**Silver Birch (*Betula verrucosa*)**
Cellulose (%)	40	39.5	45.0	41.0
Hemicellulose				
-Glucomannan (%)	16.0	17.2	3.1	2.3
-Glucuronoxylan (%)	8.9	10.4	14.1	27.5
-Other polysaccharides (%)	3.6	3.0	2.0	2.6
Lignin (%)	27.7	27.5	31.3	22.0
Total extractives (%)	3.5	2.1	2.8	3.0

1.2.2 Hemicellulose

Hemicelluloses are considered to be the second most abundant heteropolymers present in nature and are classified as xylans, mannans, arabinogalactans or arabinans depending on their sugar backbone composition (Jiang *et al*., 2006). The hemicellulose found in hardwoods consists mainly of xylans, whilst mainly glucomannans are present in softwoods (Maki *et al*., 2009). The hydrolysis of hemicelluloses in hardwoods such as *Eucalyptus* spp. involves the action of several hemicellulolytic enzymes such as endo-1-4,-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, and acetylxylan esterases (Fig. 2) (Maki *et al*., 2009). Xylanase cleaves the internal glycosidic bonds at random positions of the xylan backbone into small oligomers and is the main enzyme responsible for xylan depolymerization (Fengxia *et al*., 2008). In order to ensure accessibility of xylanolytic enzymes to xylosidic linkages in lignocellulose, microorganisms produce a system of enzymes each with specialized functions to attain greater xylan hydrolysis (Wong *et al*., 1988).

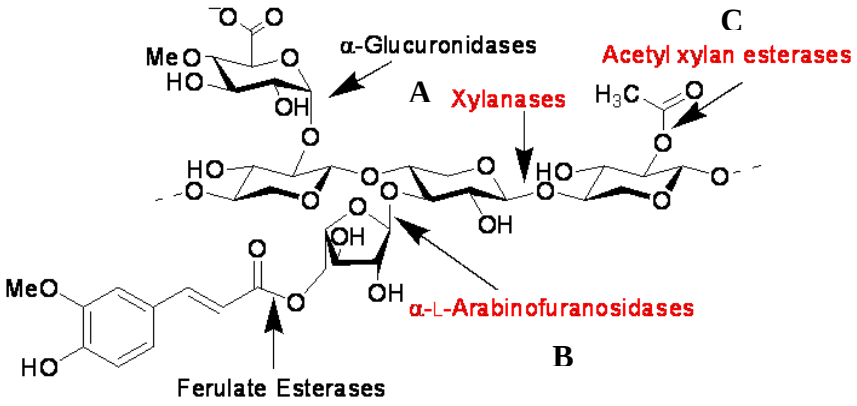

Figure 2: Multiple enzymes involved in the dissimilation of plant polysaccharides. (A) Xylanases hydrolyse the β1, 4 glycosidic bonds in xylan, (B) Arabinofuranosideases hydrolyse both the α-1,2 and 1,3 arabinofuranosyl moieties from arabinan and xylan, (C) Acetyl xylan esterases, hydrolyze the *O*-acetyl substituents at the *O*-2 position of the xylan backbone (www.york.ac.uk/depts/chem/staff/et.html).

1.2.3 Lignin

Lignin represents approximately 30% of the dry weight of softwoods and about 20% of the weight of hardwoods (Sjöström, 1993). Most of the lignin found in wood comprises of non-phenolic aryl-glycerol-β-*O*-aryl ether units. The lignin macromolecule also contains other units such as phenylcoumaran, resinal and dibenzodioxocins (Ralph *et al*., 2000). Lignin has many functions as it provides strength to the plant cell wall, assists the transport of water and inhibits the degradation of wall polysaccharides, thus providing resistance to attack by pathogens, insects and other herbivores (Hatfield and Vermerris, 2001). Certain ecological factors such as climate, plant fertilization, age of the wood and the amount of sunlight it is exposed to, affects the chemical structure of lignin (Argyropoulos and Menachem, 1997). Lignin degradation involves extracellular enzymes such as laccase, lignin peroxidase, manganese peroxidase, versatile peroxidase and H_2O_2-forming enzymes (Hatakka, 2001). Lignins are polymers made up of the phenylpropene units guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H). The specific composition of lignin varies significantly with species. In addition, lignins are divided into two main groups: guaiacyl lignins and guaiacyl-syringyl lignins (Gibbs, 1958). Ramos *et al*. (1992) reported the restriction of fibre swelling and thus enzyme accessibility by guaiacyl lignin more than syringyl lignin. Due to the bond types and heterogeneity in hardwoods, hydrolytic enzymes are not capable of degrading lignin. However, during the course of

evolution one single group of microorganisms has enhanced their ability to degrade lignin significantly and these are the basidiomycetes (Hatakka, 1994). Ubiquitous fungi and their enzymes are capable of oxidizing recalcitrant compounds as a result of their lignin-degrading enzymatic machinery in many of the reactions. These microbes have acquired an unspecific extracellular system which is able to extract one electron directly from the benzenic rings of the different lignin units (Ruiz-Dueñas and Martínez, 2009).

1.2.4 Other cell wall components

Plant cell walls contain structural proteins, phenolic polymers, enzymes and other materials which alter the chemical and physical characteristics of the wall. Extraneous material is also found in plant cell walls which include extractives and non-extractives. Extractives are generally divided into three categories, which are terpenes, resins and phenols (Gutiérrez *et al*., 1998). Sterols, triglycerides, sterol esters, fatty acids and steroid ketones are the main lipophilic groups found in fresh *Eucalyptus globulus* wood (Gutiérrez *et al*., 1998). Wood resin causes major problems in the pulping industry as the resins accumulate to form viscous masses known as pitch, which collects in the machinery and appears as dark spots on the paper. Resin content could be reduced by storing the wood as chips, rather than logs, as this increases the oxidation processes (Gutiérrez *et al*., 1998). Non-extractives include inorganic compounds such as silica, carbonates, oxalates and non-cell wall compounds such as starch, pectin and protein (Argyropoulos and Menachem, 1997; Kuhad *et al*., 1997).

The characterization of wood is usually carried out by determining specific gravity, mass density, and moisture content (Winandy, 1994; Klaassen, 2008). Specific gravity is a ratio between the weight and volume of water (at 4°C) and the wood material. It refers to the weight of the dried wood, consisting primarily of cellulose, hemicellulose and lignin. The specific gravity of such a substance is 1.5 and this value relates to all species (Francescato *et al*., 2008). Mass density refers to the ratio between weight and volume of the wood material made up of substances and voids (vascular cavities) filled with either air and/or water. Moisture content is presented as a percentage and is calculated as either the mass of water present in relation to the mass of oven-dried wood or the mass of water

present in relation to the mass of fresh wood material. The latter method is generally used in the promotion of wood fuels (Francescato *et al*., 2008).

1.3 PULP AND PAPERMAKING INDUSTRY

In South Africa trees experience a short growth cycle which is advantageous to the pulping industry as *Eucalyptus* species attain the size appropriate for pulping at nine years (Pogue, 2008). In pulp or papermaking, uniform fibre quality or attractive fibre properties are important, therefore tree species are usually grown in monoculture plantations. Different plant fibres have varied physical characteristics that determine their value in papermaking and pulping. Advances in planting strategies and hybridization have allowed for the propagation of faster-growing trees with disease resistance and cold tolerance (Ince, 2004).

The major steps in pulp manufacturing are: (i) raw material preparation; such as wood debarking and chip production; (ii) pulp manufacturing; (iii) pulp bleaching; (iv) manufacturing of products; and (v) fibre recycling. Pulp mills and paper mills may function independently or as integrated operations (World Bank, 1998).

1.3.1 Wood species used in the pulp and paper industry

There are mainly three species of trees used in the South African plantation forestry: *Eucalyptus*, pine and wattle. The growth rotations of these trees differ based on species of tree, surrounding environmental conditions and management practices (Pogue, 2008). The majority of the South African forestry industry utilizes exotic tree species such as *Eucalyptus*, *Pinus* and *Acacia* (Viljoen *et al*., 1992; van Staden *et al*., 2004). The most extensively cultivated hardwood in the world is *Eucalyptus*. They are planted widely as exotic plantation species in tropical and subtropical zones throughout South America, Australia, Asia, and Africa, and in the more temperate regions of North and South America, Australia, and Europe (FAO, 2005). The propagation of *Eucalyptus* has increased over the past few decades, particularly in tropical countries which have regions of faster development (Rockwood *et al*., 2008). *Eucalyptus* spp. are popular in forest plantations due to their rapid growth rate, excellent wood qualities and adaptability to

varying environmental conditions (Turnbull, 2000). *Eucalyptus* is also used in pulp milling for the production of dissolving pulp, smoother paper, corrugated cartons and fluting. Pine species is softwood used in sawmilling for timber and in pulp milling for the manufacturing of newsprint, packaging and magazines.

Species trials have identified the most valuable commercial eucalypts worldwide, the majority of which come from the subgenus *Symphomyrtus* (Potts and Dungey, 2001). Several species that were formerly regarded as important have fallen into disapproval, mainly due to susceptibility to damage by pests and diseases or inadequate environmental adaptation, poor growth performance and inferior wood properties (Low and Shelbourne, 1999; Clarke 2000). In South Africa this is predominantly true of species classified in the subgenus *Monocalyptus*. Species such as *E. fraxinoides*, *E. regnans*, *E. fastigata*, *E. oreades* and *E. elata* have poor endurance in the summer rainfall regions due to attack by *Phythophthora* sp. (Clarke and Jones, 1998).

1.3.1.1 *Eucalyptus grandis*

Historically, *E. grandis* has been the most important hardwood for the South African forestry industry. However, an increasing demand for hardwoods particularly for the pulp and paper industry has led to the expansion of hardwoods into colder sites where *E. grandis* does not survive (Kunz and Gardner, 2001). According to Gardner and Swain (1996) *E. grandis* and *Acacia mearnsii* are the most susceptible commercial hardwood species with *E. nitens* being the most resistant to snowfalls. *E. grandis* grows in humid to sub-humid conditions with a low incidence of frost (Boland *et al*., 1980). This species is ideally suited to sites in the summer rainfall regions of South Africa with a mean annual temperature (MAT) of greater than 17°C and a mean annual precipitation (MAP) greater than 900 mm for optimum growth (Herbert, 2000). *E. grandis* is classified as sub-tropical and is not suitable for areas where frost and snow events occur. *E. grandis* is susceptible to various forms of diseases such as *Crysoporthe austroafricana* canker particularly in the first two years following establishment and *Coniothyrium* sp. canker which occurs as lesions on the stem. The bark of this species strips easily throughout the year but is susceptible to drought which negatively affects the stripping ability. It also has a lower

density relative to all other commercial eucalypts, but has both good kraft and dissolving pulp properties (Table 2) (Clarke, 2000).

1.3.1.2 *Eucalyptus nitens*

In South Africa *E. nitens* is ideally suited to cooler sites in the summer rainfall regions of the country with MAT not greater than 13.1-15°C and MAP above 810-899 mm for optimum growth (Herbert, 2000). This species is classified as frost tolerant, but is not as hardy as *E. macarthurii* which exhibits high snow tolerance. *E. nitens* is susceptible to various forms of leafspot (*Mycosphaerella* sp.) in its juvenile state. Diseases caused by *Endothia* and *Botrysphaeria* spp. appear in several environmental stress factors such as drought, frost or hail. *E. nitens* in recent years has become susceptible to attacks from *Coryphodema tristis*, an indigenous cossid moth (Boreham, 2004). This species strips relatively easily and has good kraft pulping properties (Clarke, 2000).

1.3.1.3 *Eucalyptus dunnii*

E. dunnii grows better than *E. grandis* in cooler sites and has better frost tolerance. It is ideally suited to sites in the summer rainfall regions of South Africa with MAT greater than 15.5°C and MAP between 822–925 mm for optimum growth (Schönau and Gardner, 1991). *E. dunnii* is classified as mildly drought tolerant, susceptible to frost and snow damage. It is susceptible to *Gonipterus scutellatus* (snout beetle) particularly at high altitudes (>1 300 m above sea level) and during periods of stress such as drought. Following introduction *E. dunnii* has remained relatively disease free with a few recorded cases caused by *Botryosphaeria* sp. which appear following environmental stresses such as drought, frost or hail. This species strips relatively easily and has above average density with a range of pulping properties suitable for both dissolving and kraft processes (Clarke, 2000).

1.3.1.4 *Eucalyptus macarthurii*

Frost damage is severe in the Highveld of Mpumalanga and selected areas in KwaZulu-Natal (KZN), particularly in the valleys and drainage areas. Some species may be entirely scorched and drop leaves but have the ability to recover in the spring. This is

characteristic of *E. macarthurii*, one of the most frost-tolerant species cultivated in South Africa. It is ideally suited to sites in the summer rainfall regions of the country with MAT between 13.1°C and 16°C and a MAP range of 738-864 mm for optimum growth (Gardner and Swain, 1996; Herbert, 2000). *E. macarthurii* is classified as cold tolerant and the most frost resilient of all the commercial Eucalypt species, but is vulnerable to stem breakage following mild to heavy snowfalls. This eucalypt is susceptible to various forms of *Phythophthora* sp. especially in the first two years following establishment and remains fairly disease free due to its thick bark. *E. macarthurii* strips easily during summer but with difficulty during winter and has above average density. This wood species has low pulping properties for both kraft and dissolving pulp (Clarke, 2000).

1.3.1.5 *Eucalyptus smithii*

E. smithii is ideally suited to deep well drained soils on cool sites in the summer rainfall regions of South Africa with a MAT not greater than 15-17°C and MAP above 819-936 mm for optimum growth (Schönau and Gardner, 1991; Herbert, 2000). *E. smithii* is categorized as cold tolerant and not frost hardy with moderate snow tolerance. This eucalypt is prone to infection by various forms of *Phythophthora* sp., generally in the first two years of growth. In subsequent years *Botrysphaeria* sp. will appear as a result of environmental stresses such as drought, frost or hail. This species strips fairly easily during summer and has above average density and good pulp properties.

1.3.1.6 Hybrids

Innovation of Eucalypt hybrids has become a major element of plantation forestry, specifically in the sub-tropics, tropics and to a lesser degree in the more temperate zones. Designing hybrids that combine complementary traits is currently the focus of hybrid development (Potts and Dungey, 2001). Inter-specific *Eucalyptus* hybrids have been developed for any one of three reasons: to combine desired traits of two species; to promote hybrid vigour (heterosis); or to enhance adaptability of a Eucalypt species to areas which are marginal for the parent species. Some of the more prevalent hybrid combinations include *E. grandis* × *E. urophylla* (combining good growth with *Coniothyrium* tolerance), *E. grandis* × *E. camaldulensis* (combining good growth with

drought tolerance) and *E. grandis* × *E. nitens* (combining good growth with cold tolerance and rooting ability) (Verryn, 2000).

Table 2: The relative expression of traits and important commercial characteristics for selected *Eucalyptus* species (Clarke and Jones, 1998; Morris and Pallett, 2000)

Species	Improved Genetic Stock	Tolerance			Disease Resistance	Insect Tolerance	Growth Rate	Bark Stripping	Density	Wood Properties		
		Cold	Snow	Drought						Solid	Kraft	Dissolving
E. dunnii	H	L	L	L	L	H	M	M	M	L	M	M
E. grandis	H	L	L	M	M	L	H	H	L	M	M	M
E. nitens	M	M	H	L	L	M	-	M	-	M	H	L
E. macarthurii	H	H	L	M	M	M	-	L	H	M	L	L
E. smithii	M	M	M	M	L	M	H	M	H	L	M	H
E. grandis*×*E. camaldulensis	M	L	L	H	M	H	M	H	M	L	L	M
E. grandis*×*E. urophylla	M	L	L	L	M	H	H	H	M	L	L	M

Note: H = high M = moderate L = low

1.3.2 Wood chip production

The trunk of a tree is made up of bark on the outside and bast and cambium inside which form the growth tissue. It can be used in the production of pulp, but only after it has been debarked. Once the tree has been debarked it is sent to the chipper to be processed into uniform wood chips (Sappi-Chemical Cellulose, 2007). Chipped wood material is easier to handle, transport and store compared to round wood, thus increasing the outdoor storage of wood chips in piles in the past 58 years. Once harvested, the wood is debarked, chipped at the mill and stored in piles within a few days. Therefore the wood in the piles is still wet and may hold over 50% by weight water (Brown *et al*., 1994).

The properties of stored materials are known to change due to processes such as hydrolysis, autoxidation or microbial degradation (Sjöström, 1993). Among all the

volatile extractives that are easily released during storage at low temperatures, terpenes represent the largest part (Lehtikangas, 2001). Stored chips are also reported to contain an immense amount of reasonably large mineral particles. It has been reported that following storage, woodchips with higher densities tend to have enhanced durability (Lehtikangas, 2000).

1.3.2.1 Factors affecting wood chip piles

There are multiple factors that may influence the degradation of wood chips. Deterioration may be dependent on the properties of the material, such as chemical structure, moisture content and availability of nutrients or on the form of storage, such as size of the pile, its compacting or covering (Lehtikangas, 2000; Hogland and Marques, 2003). Moisture is present in wood in several forms, such as physisorbed, chemosorbed, bulk and surface. The availability of water to microbes is essential in promoting fermentation and initiating the growth of bacteria (Li *et al*., 2006). There have been numerous reports of major losses of chip piles due to high temperatures and combustion (Fuller, 1985; Tansey, 1971; Ferrero *et al*., 2009). The spontaneous combustion of wood chip piles has been ascribed to the heat energy released by microbial fermentation (Li *et al*., 2006). During the first five to seven days of storage the living cells in the wood remain viable and continue to respire resulting in heat being generated, with the highest temperature reported at the centre of the pile (Fuller, 1985; Nurmi, 1999). When temperatures of 60 - 70°C are reached, a chemical reaction takes place in which the acetyl group attached to each hemicellulose molecule is cleaved, forming acetic acid. The rise in acidity and heat causes the wood to darken and ultimately disintegrate as if burned (Fuller, 1985). The use of these wood chips in the ‘cooking’ process result in low yields and high pulpscreen rejects. Fermentation becomes inactive at temperatures higher than 70°C, as most bacteria and fungi are not able to survive at these temperatures. Thus, any further increase in temperature to above 100°C to the ignition temperature (minimum temperature at which wood will spontaneously burn independent of a heat source) is attributed to oxidation (Li *et al*., 2006).

1.3.2.2 Management of piles

Fuller (1985) created a protocol for chip pile management by identifying the causes and recommending ways to avoid deterioration. Pile height should be maintained below 15 m, thus preventing compaction and allowing heat produced during microbial metabolism to be released. Tractor spreading of recently delivered chips should be avoided, as this could create fines which look like sawdust. Modern mills have chip pile management equipment facilitating rotation of the pile at regular periods (Fuller, 1985). It has been recommended that species with different deterioration rates should be mixed as needed, so that chips that have a high deterioration rate are not clumped together in the pile creating a large zone with high temperatures. The addition of fine particles such as sawdust and shavings should be avoided as this adds to compaction and the trapping of heat (Fuller, 1985; Hogland and Marques, 2003). The temperature of the pile should be monitored regularly, so that heating problems may be identified early (Fuller, 1985). To avoid spontaneous combustion or heating, material must not be damp and not stored in large volumes (Li *et al*., 2006).

1.3.2.3 Preservation techniques

There are currently several methods available to prevent unfavourable degradation of wood chips in piles, such as chemical and biological preservation of wood, management of pile height and compaction, mixing of species of different deterioration rates and monitoring pile temperature (Fuller, 1985; Ejechi, 2003). The degradation of carbohydrates by selective microorganisms has been known to increase the lignin content of wood after storage, therefore decreasing the quality of paper produced and increasing the use of chemicals (Lentikangas, 2001). A major factor in the use of chemicals to treat wood is that the fungicidal composition must be active for a minimum of 20 days and up to 60 days and the concentration and volume of the fungicide applied must be economically feasible (Brown *et al*., 1994). It was found that thiocarbonates were able to preserve piles for at least 20 days and in some cases up to 60 days. Thiocarbonates also acted as antioxidants and were able to control temperature increases from non-biological sources as well (Brown *et al*., 1994). An alternative to this method is the distribution of evaporable ammonium salts close to the wood in a restricted space, so that the salts create

an atmosphere around the moist wood thus preventing the growth of microorganisms (Häger, 1986).

In order to develop proactive strategies to ensure the quality of wood chips, a systematic study needs to be conducted to establish parameters such as: type of wood (tree species), chemical composition of wood and the environment in which it was grown, the natural microflora present and the effect that each of these parameters has on the storage quality of wood chips.

1.4 BACTERIAL DEGRADATION OF WOOD

Bacteria are known to affect wood permeability and damage wood structure. They work synergistically with soft-rot fungi to predispose wood to fungal attack. The cellulolytic and pectinolytic enzyme systems of bacteria play a significant part in structural changes in wood. Cellulases produced by bacteria alter the permeability of wood by opening up the crystalline arrangement of the cellulose as a target for further diffusion of cellulolytic enzymes (Clausen, 1996). Common cellulase producers include *Paenibacillus*, *Bacillus*, *Cellulomas flavigena*, *Terendinibacter turnerae* (Maki *et al*., 2009). Bacterial pectinases target the membrane of the bordered pit, resulting in complete degradation of the pit membrane (Clausen, 1996). The hemicellulose in hardwoods contains mostly xylans, therefore a variety of enzymes such as endo-1-4,-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, as well as acetylxylan esterases are required for its degradation (Maki *et al*., 2009). Xylanase removes xylan, which improves the removal of lignin by other microorganisms (Savitha *et al*., 2009). Qualitative screening methods are generally used to estimate the activity of the above mentioned enzymes (De Koker *et al*., 2000; Singh *et al*., 2000; Bucher *et al*., 2004). Bacteria that degrade wood structures can be divided into two groups, erosion or tunnelling bacteria (Clausen, 1996).

1.4.1 Erosion bacteria

Erosion bacteria are rod or spherical in shape, 1-4 µm long, 0.5-1 µm thick, gram-negative cells that lack flagella, but possess a thick slime layer and are motile *via* gliding. The slime layer aids in the attachment of the bacterial cell to the cell wall and those that

are able to attach in this way are capable of degrading wood. These bacteria are known to target the cellulose rich S_2 layer (Klaassen, 2008). Erosion bacteria are usually found in environments with low oxygen concentrations (Gelbrich *et al*., 2008). It has been suggested that erosion bacteria may be stimulated by the presence of other bacteria, thus implying that the associated bacteria may be supplying stimulating growth factors (Nilsson *et al*., 2008). Erosion troughs are divided into two types: (1) shallow, surface troughs in the S_3 layer, commonly caused by bacilli; and (2) deep troughs that advance from the lumen to the secondary cell wall, frequently caused by cocci (Greaves, 1971). The effects of erosion bacteria and different degradation patterns produced may be investigated by using a light microscope, scanning electron microscope or transmission electron microscope (Gelbrich *et al*., 2008; Klaassen, 2008). The use of Fourier transform infrared spectroscopy (FTIR) has been reported to be an excellent device in the detection of the level of bacterial decay in wood (Gelbrich *et al*., 2008).

1.4.2 Tunnelling bacteria

Tunneling bacteria are characteristically found in surroundings comparable to that of soft-rot fungi (Gelbrich *et al*., 2008). However, it has been proposed that anaerobic microniches occur in wood structures due to the depletion of oxygen by respiring aerobic microbes (Clausen, 1996). Tunnelling bacteria are capable of attacking all cell wall layers. Bacteria in the cell lumen initially gain entry into the cell wall at restricted sites, which are the sites of their attachment and then proceed to degrade it by a tunnelling action. The middle lamellae of the cell which is highly lignified, is also infiltrated by tunnelling bacteria and no wall residue is left behind indicating that these microorganisms are capable of metabolizing lignin (Kim and Singh, 2000). The transfer and distribution of invading organisms must rely on motility or the synergistic effects of multiple enzymes in order to infiltrate the wood (Clausen, 1996). This type of decay results in tunnels with crescent-shaped bands in the wood cells which can be examined using transmission electron microscopy (Clausen, 1996; Kim and Singh, 2000). Rogers and Baecker (1991) were the first scientists to isolate and identify *Clostridium xylanolyticum* (Table 3) as one of the bacteria accountable for tunnelling decay.

Table 3: Bacteria that colonize *Eucalyptus* species

SPECIES	CAUSES	REFERENCE
Clostridium xylanolyticum	Tunnelling decay, produces xylanases	Rogers and Baecker (1991)
Ralstonia solanacearum	Causes bacterial wilt in *Eucalyptus*	Dianese *et al*. (1990)
Xanthomonas eucalypti	Causes bacterial dieback in *Eucalyptus*	Truman (1974)
Xanthomonas campestris	Causes shoot blight	Wardlaw *et al*. (2000)
Erwinia sp.	Infects *Eucalyptus*	Coutinho *et al*. (2002)
Pantoea ananatis	Infects *Eucalyptus*	Coutinho *et al*. (2002)
Agrobacterium tumefaciens	Causes crown gall	Wardlaw *et al*. (2000)

Common bacteria that infect *Eucalyptus* trees are *Ralstonia solanacearum* which causes bacterial wilt and *Xanthomonas eucalypti* which causes bacterial dieback (Truman, 1974; Dianese *et al*., 1990). A tree infected with bacterial blight displays symptoms such as tip dieback and leaf spots on young leaves. The leaf petioles eventually become necrotic, resulting in abscission of the leaves. In the advanced phase of this disease, the tree exhibits a scorched appearance and after multiple infections becomes stunted (Coutinho *et al*., 2002). This type of attack on *Eucalyptus* is more dominant in areas of South Africa where the temperature is relatively low (about 20 to 25°C) and the humidity is relatively high. Coutinho and colleagues (2002) reported the first incidence of bacterial blight and dieback induced by *Pantoea ananatis* on *Eucalyptus* in South Africa. In 1977 a *E. grandis* × *E. camaldulensis* (GC) hybrid in Zululand, KZN was found to be infected with *R. solanacearum*, making this the first account of bacterial wilt on *Eucalyptus* in South Africa (Coutinho *et al*., 2000).

Bacteria have been found to tolerate preservatives at levels that are commonly used to prevent fungal growth. By removing competition from fungi, the use of these preservatives indirectly improves bacterial growth rates (Clausen, 1996). The presence of thermophilic and thermotolerant bacteria is dominant in wood chip piles as temperatures reach up to 45-50 °C, thus preventing the growth of basidiomycetes. The outer layers of the chip pile are cooler than the inner layers; therefore bacteria found in the outer layers are in association with the fungi present (Clausen, 1996).

1.5 FUNGAL DEGRADATION OF WOOD

Three fungal wood types (Table 4) have been reported: soft rot, white rot and brown rot (Bucher *et al*., 2004). White-rot fungi cause rapid and widespread decay of all wood components by enzymatic degradation, and wood bleaching is also observed due to lignin removal. Brown-rot fungi bring about rapid cellulose and hemicellulose decay by non-enzymatic oxidation and with limited amounts of lignin degradation. Soft-rot fungi cause degradation of cellulose and hemicellulose in the surface layers of wood with little lignin degradation observed (Risna and Suhirman, 2002; Urairuj *et al*., 2003; Bucher *et al*., 2004). Clausen (1996) has suggested that the presence of bacteria may be favorable to fungi as their metabolic products may act as growth factors. It has also been proposed that due to the low nitrogen concentrations in wood, fungi obtain their vital nitrogen supply from nitrogen-fixing bacteria, which in turn utilize carbohydrates released by fungi (Cowling and Merrill, 1966). *Trichoderma reesei* is a fungus that is well known for its cellulolytic and xylanolytic capabilities (Den Haan and Van Zyl, 2003).

1.5.1 White-rot

White-rot fungi belong to the basidiomycetes family amongst others, and their enzymes are known to be proficient lignin degraders (Blanchette and Reid, 1986; Temp *et al*., 1998). White-rot fungi produce lignin peroxidases, manganese (Mn) peroxidases and laccases in order to oxidize lignin (De Koker, 1998). There are generally two types of white-rot: (i) simultaneous white-rot which degrades all cell wall components and (ii) selective white-rot which only removes the lignin throughout the wood cell wall leaving the cellulose intact (Blanchette, 1984; Srebotnik and Messner, 1994). White-rot fungi have been known to preferably attack and degrade hardwood species. *Ceriporiopsis subvermispora* has been proposed as an excellent example of a selective lignin degrader (Akhtar *et al*., 1998; Blanchette *et al*., 1992). During wood biodegradation, *C. subvermispora* produces oxidative enzymes, with the main enzyme being Mn-dependent peroxidase followed by laccase (Tanaka *et al*., 2009). *Phanerochaete chrysosporium* is one of the most commonly studied white-rot fungi, and has great potential in biopulping (De Koker *et al*., 1998). These white-rot fungi are vital because they may be used for the removal of lignin from lignocellulosic materials that would assist in biopulping,

biobleaching and detoxification of environmental pollutants (Blanchette and Reid, 1986; Temp *et al*., 1998).

1.5.2 Brown-rot

Brown-rot fungi are known to principally degrade the polysaccharides in wood; however a small amount of lignin alteration does take place. Attack by brown-rot fungi results in a brittle wood appearance and a residual brown substance (rich in tannin and extractives) on the wood. Most brown-rot fungi prefer to degrade softwood species (Bucher *et al*., 2004). Brown-rot fungi are known to cause the most extensive damage to wood by rapidly depolymerizing the polysaccharide component (Valášková and Baldrian, 2006). Although brown-rot basidiomycetes are able to cause widespread lignin degradation, little lignin is actually removed, most likely due to deficiencies in ligninolytic peroxidases or oxidases. Brown-rot fungi are however, able to remove all the cellulose and hemicelluloses from wood, resulting in wood containing primarily lignin (Valášková and Baldrian, 2006).

1.5.3 Soft-rot

Most soft-rot fungi are ascomycetes and are comprised of many economically significant species from genera such as *Ophiostoma* and *Ceratocystis* (De Beer *et al*., 2003). *Ophiostoma* sp. and *Ceratocystis* sp. have previously been linked with disease and bluestain of commercial *Eucalyptus* trees, timber as well as pulpwood (De Beer *et al*., 2003; Roux *et al*., 2004). These types of fungal infestations mainly occur due to wounds in the bark and sapwood of trees, usually initiated by animal damage or commercial harvesting methods (Grobbelaar *et al*., 2010). *Ceratocystis* sp. are also known to cause root and fruit rots, stem cankers and vascular wilts (Roux *et al*., 2004). *C. fimbriata* has been reported as a common pathogen of forest plantations in South Africa (Roux *et al*., 2004). Examples of soft-rot fungi include *Phialophora hoffmannii* and *P. fastigiata*. Soft-rot fungi penetrate wood by erosion of the wood cell wall by releasing enzymes from the hyphae on the lumen surface of the wall or by the creation of cavities around the hyphae in the S_2 layer of the cell wall (Hale and Eaton, 1985).

Table 4: Fungi that colonize *Eucalyptus* and *Pinus* trees

SPECIES	HOST	REFERENCE
Botryosphaeria dothidea	*Eucalyptus* sp.	Smith *et al*. (1996)
Ceriporiopsis subvermispora	*Eucalyptus* sp.	Akhtar *et al*. (1998)
Ceratocystis fimbriata	*Eucalyptus* sp.	Roux *et al*. (2000)
Ceratocystis moniliformis	*Eucalyptus grandis*	Roux *et al*. (2004)
Colletotrichum gloeosporioides	*Eucalyptus* sp.	Viljoen *et al*. (1992)
Coniothyrium zuluense	*Eucalyptus* sp.	Wingfield *et al*. (1997)
Cryphonectria cubensis	*Eucalyptus* sp.	van Staden *et al*. (2004)
Cryphonectria eucalypti	*Eucalyptus* sp.	Gryzenhout *et al*. (2003)
Cylindrocladium scoparium	*Eucalyptus* sp.	Viljoen *et al*. (1992)
Harknessia hawaiiensis	*Eucalyptus* sp.	Viljoen *et al*. (1992)
Idiocercus australis	*Eucalyptus* sp.	Crous *et al*. (1990)
Lasiodiplodia theobromae	*Eucalyptus* sp.	Crous *et al*. (2000)
Mycosphaerella spp.	*Eucalyptus* sp.	Smith (2006)
Mycosphaerella Africana	*Eucalyptus viminalis*	Pavlic *et al*. (2007)
Neofusicoccum eucalyptorum	*E. grandis* and *E. nitens*	Pavlic *et al*. (2007)
Neofusicoccum luteum	*Eucalyptus* sp.	Pavlic *et al*. (2007)
Neofusicoccum parvum	*Eucalyptus* sp.	Slippers *et al*. (2004)
Ophiostoma piliferum	*Pinus radiate*	de Beer *et al*. (2003)
Ophiostoma quercus	*Eucalyptus* sp.	Grobbelaar *et al*. (2010)
Ophiostoma tsotsi	*Eucalyptus* sp. and *Pinus* sp.	Grobbelaar *et al*. (2011)
Phaeoseptoria eucalypti	*Eucalyptus* sp.	Viljoen *et al*. (1992)
Phanerochaete chrysosporium	*Eucalyptus* sp. and *Pinus* sp.	De Koker (1998)
Phytophthora cinnamomi	*Eucalyptus* sp.	Viljoen *et al*. (1992)
Pleurotus ostreatus	*Eucalyptus* sp.	Cohen *et al*. (2002)
Sphaeropsis sapinea	*Eucalyptus* sp.	van Staden *et al*. (2004)
Sphaerotheca pannosa	*Eucalyptus* sp.	Viljoen *et al*. (1992)
Sporothrix eucalypti	*Eucalyptus grandis*	De Beer *et al*. (2003)
Stereum spp.	*Eucalyptus* sp.	De Koker *et al*. (2000)

1.6 FUNGAL DEGRADATION OF LIGNIN

The removal of lignin is commercially important as this is required to produce high-grade paper (Miller, 1999). Lignin is regarded as an obstruction that must be removed before the cellulose contained in wood chips is made accessible to other enzymes (De Koker *et al*., 1998). The mechanism of lignin degradation involves oxidation of lignin by manganese peroxidases (MnP) and lignin peroxidase (LiP) (De Koker *et al*., 2000; Bucher *et al*., 2004). It has been suggested that phenol oxidases such as laccase and tyrosinases are also involved in this process (De Koker *et al*., 2000). Gelbrich *et al*. (2008) reported that increased decay of wood resulted in increased lignin levels, as well as increased nitrogen and phosphorus levels.

1.6.1 Lignin peroxidase (LiP)

LiPs are regarded as the main catalyst in the fungal breakdown of lignin (Srebotnik *et al*., 1994). LiP found in *Phanerochaete chrysosporium* is a heme-containing glycoprotein, which is produced during secondary metabolism as a reaction to nitrogen limitation (Breen and Singleton, 1999). These enzymes apply free radical chemistry to cleave the propyl side chain of lignin substructures. Remarkably, they are able to cleave the recalcitrant nonphenolic units that make up approximately 90% of lignin (Srebotnik *et al*., 1994). Although, not all white-rot fungi are able to produce this enzyme, yet they are still able to degrade lignin found in wood (Srebotnik and Messner, 1994).

1.6.2 Manganese peroxidase (MnP)

MnP is a heme-containing glycoprotein found in *P. chrysosporium*, and is able to degrade lignin in wood (Hofrichter, 2002). MnPs oxidize Mn^{2+} to Mn^{3+} and can be stimulated by lactate, which most likely acts by chelating Mn^{2+} to form stable complexes with a high redox potential. The Mn^{3+} in turn oxidizes phenols to phenoxy radicals. MnP is susceptible to high hydrogen peroxide concentrations, as it initiates a catalytically inactive oxidation state (Hofrichter, 2002). It has been suggested that Mn^{3+} oxalate, which is a product of the MnP cycle, may stimulate lipid peroxidation that successively results in lignin degradation through the action of peroxyl or acyl radicals (Vicentim and Ferraz, 2007). A reduction in the amount of 4-acetoxycinnamyl acetates produced in the

premature stage of decay may be valuable in predicting the extent of lignin degradation (Vicentim and Ferraz, 2007). The MnP cycle produces low molecular weight diffusible oxidizing agents that are proficient in wielding a response over a distance from the enzyme (Breen and Singleton, 1999).

1.6.3 Laccase

Laccases are described as multi-copper-containing enzymes that oxidize phenolic compounds. *P. chrysosporium* does not encode conventional laccases, however four multicopper oxidase (MCO)s are thought to have a role in extracellular oxidations (Martinez *et al*., 2004). Laccase could interact with phenolic compounds of lignin directly or if a 'mediator' such as a co-substrate is available and may respond to an extensive range of substrates (Breen and Singleton, 1999). Moldes *et al*. (2008) compared several natural and synthetic mediators in terms of efficiency in laccase-assisted bleaching of Eucalypyus kraft pulp. They reported that syringaldehyde was the only natural mediator of those tested that enabled improved pulp exploitation characteristics such as delignification and brightness, although its efficiency was lower compared to the synthetic mediators 1-hydroxybenzotriazole, violuric acid. It has been reported that the addition of nitrogen sources and glucose to wood-containing cultures of *C. subvermispora*, the production of laccases is induced during the initial stages of wood decay (Vicentim and Ferraz, 2007).

1.7 MICROBIAL DEGRADATION OF CELLULOSE

Hydrolysis of cellulose is performed by cellulases which are highly specific enzymes (Béguin and Aubert, 1994). The byproducts of hydrolysis are usually reducing sugars such as glucose. Bacteria (e.g.: *Clostridium*, *Cellulomonas*, *Bacillus*, *Erwinia* and *Streptomyces* spp.) and fungi (*Sclerotium rolfsii*, *Phanerochaete chrysosporium*, *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* spp.) are capable of producing cellulases for the degradation of lignocellulosic substrates (Fan *et al*., 1987; Bisaria, 1991; Duff and Murray, 1996; Shin *et al*., 2000). Generally, cellulases are bimodular proteins with a large catalytic and small carbohydrate binding molecule (CBM) connected by a short highly glycosylated protein sequence (Parry *et al*., 2002). There are

three major groups of cellulases implicated in the hydrolysis process: endoglucanase, exoglucanase and β-glucosidase (Coughlan and Ljungdahl, 1988). Endoglucanases (1,4-β-D-glucan-4-glucanohydrolases EC 3.2.1.4) randomly hydrolyze internal bonds in the cellulose polysaccharide chain, releasing new terminal ends (Pérez *et al*., 2002). Exoglucanases (1,4-β-D-glucan glucanohydrolases EC 3.2.1.74) act on the existing or endoglucanase-generated chain ends, liberating either glucose or cellobiose as major products (Lynd *et al*., 2002). Exoglucanases are the only enzymes that efficiently degrade crystalline cellulose by peeling cellulose chains from the microcrystalline structure (Pérez *et al*., 2002). β-Glucosidases (EC 3.2.1.21) degrade cellobiose molecules to produce two glucose molecules (Pérez *et al*., 2002). Factors that may affect enzyme activity include cellulase activity, substrates, and reaction conditions such as temperature pH, etc. (Sun and Cheng, 2002).

1.8 MICROBIAL DEGRADATION OF HEMICELLULOSE

Hemicellulases are classified based on to the substrates they act on, by the bonds they cleave and arrangements of product formation (Jeffries, 1994). The degradation of hemicellulose requires hydrolysis of non-xylose substituents from the xylan backbone in conjunction with endoxylanases and β-xylosidases (Johnson *et al*., 1989). Xylanase cleaves the internal glycosidic bonds at random sites of the xylan backbone into to small oligomers, and is the key enzyme responsible for xylan depolymerization (Fengxia *et al*., 2008). To ensure accessibility of xylanolytic enzymes to xylosidic linkages in lignocellulose, microorganisms produce a system of enzymes that may include multiple xylanases; β-xylosidase and accessory enzymes, each with specific functions, to attain greater xylan hydrolysis (Wong *et al*., 1988). Several bacteria, yeasts and fungi are capable of producing a range of xylanases and the nature of these enzymes varies among different organisms (Kinegam *et al*., 2007). Xylanases may be characterized into two groups; endo-1,4-β-xylanases (EC 3.2.1.8, D-xylan xylanohydrolase) and exo-1,4-β-xylanases (EC 3.2.1.37, D-xylanohydrolase) (Christakopoulos *et al*., 1996). Endoxylanases promote the hydrolysis of internal bonds of the xylan backbone, whilst exoxylanases demonstrate a preference for groups at the termini of xylan chains (Christakopoulos *et al*., 1996; Oakley *et al*., 2003). Classification of β-D-xylosidases (EC

3.2.1.37, 1, 4-β-D-xylan xylohydrolase) is based on their affinities for xylobiose and larger xylo-oligosaccharides (Biely, 1985). 1,4-β-xylosidase hydrolyses short xylo-oligomer chains into xylose. The activity of this enzyme is favored in the removal of monomers from the non-reducing end of the xylooligomer with an increasing attraction with decreasing degree of polymerization (Coughlan *et al*., 1993). However, this enzyme is susceptible to inhibition by the xylose end-product (Poutanen *et al*., 1991). α-D-Glucuronidase (EC 3.2.1.139) hydrolyses the α-1,2-glucosidic linkage with the xylopyranose unit to release D-glucuronic acid. In the xylan of hardwoods, D-glucuronic acid is found in the form of 4-*O*-methyl ether (Subramaniyan and Prema, 2003). α-Arabinofuranosidase are known to yield arabinose from arabinans, arabinoxylans and arabinogalactans. This enzyme shows preference to an arabinose-linked oligosaccharides rather than the xylan chain itself (Coughlan *et al*., 1993; Subramaniyan and Perma, 2003). Acetyl xylan esterases liberates the *O*-acetyl group generally found in hardwood xylan. These esterases are reported to act on both the xylan polymer and xylooligomers.

1.9 METHODS FOR IDENTIFYING AND MONITORING MICROBIAL POPULATIONS

The structure of the wood and its degradation patterns may be determined by staining sections of wood and viewing under a light microscope (Klaassen, 2008). In order to observe fungal growth and its impact on wood, scanning electron microscopy is generally employed. The presence of microorganisms may also be detected by assaying for particular enzyme activities (Maki *et al*., 2009; Savitha *et al*., 2009). Microorganisms in environmental niches form complex consortiums which require specific nutrients, resulting in only a minute percentage of microorganisms cultivated under laboratory conditions (Schabereiter-Gurtner *et al*., 2001). Limitations of culture based techniques in ecological explorations have often been emphasized (e.g. Bridge and Spooner, 2001; Zak and Visser, 1996), as the data provide only a selective and consistently prejudiced, observation of diversity (Anderson and Cairney, 2004). There have been numerous reports on molecular methods providing a more complete examination of the microbial community than traditional culturing techniques (Schabereiter-Gurtner *et al*., 2001). Culture-independent techniques such as analysis of DNA and/or RNA extracted directly

from environmental samples, has been the decisive factor in evolving microbial ecology (Muyzer and Smalla, 1998; Ranjard *et al*., 2000; Duong *et al*., 2006; Oros-Sichler *et al*., 2006.

In this technological age genetic fingerprinting has dominated microbial community analyses. Genetic fingerprinting requires direct analysis of PCR products amplified from environmental DNA to generate a profile of microbial communities present (Muyzer, 1999). The application of molecular techniques such as 16S rRNA amplification sequencing and/or Restriction Fragment Length Polymorphisms (RFLPs) is now common practice in investigating microbial diversity and examining the organization of microbial communities (Muyzer and Smalla, 1998). Other techniques include Denaturing/Temperature Gradient Gel Electrophoresis (D/TGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP), Amplified rDNA Restriction Analysis (ARDRA) and Ribosomal Intergenic Spacer Analysis (RISA) (Anderson and Cairney, 2004, Rastogi and Sani, 2011) which produce complex community profiles that does directly reveal taxonomic composition but permits analysis and comparisons of community composition. The variation of profiles between samples reveals differences in community composition and abundance of individual microbial populations within a community. Phylogenetic information about particular members of the community is usually achieved with cloning and DNA sequencing (Kent and Triplett, 2002).

1.9.1 Amplified rDNA Restriction Analysis

ARDRA is based on DNA sequence variations present in PCR-amplified 16S rRNA genes. Following PCR amplification, 16S rRNA sequences are digested by restriction enzymes and analyzed by gel electrophoresis or amplified genes are analyzed by cloning and restriction digestion or sequencing (Smit *et al*., 1997). Generally, tetracutter restriction endonucleases (eg: *Alu*I and *Hae* III) are employed in the digestion of the PCR product amplified from environmental DNA (Rastogi and Sani, 2011). In addition to monitoring of microbial communities over time and fluctuating environmental conditions, ARDRA is also used for the identification of unique clones and estimating operational taxonomic units (OTUs) in environmental clone libraries based on restriction

profiles of the clones. The limitation of this technique is that resolution of profiles using agarose/PAGE is at times difficult (Smit *et al*., 1997). Multiple studies have implemented this technique for the analysis of microbial communities in soil (Martin-Laurent *et al*., 2001), groundwater (Cho *et al*., 2003), activated sludge (Gich *et al*., 2000), and trees (Procópio *et al*., 2009).

1.9.2 Terminal Restriction Fragment Length Polymorphism

T-RFLP utilizes a similar technique to ARDRA except for the inclusion of one 5' fluorescently labeled primer in the PCR reaction. PCR products are digested with restriction enzymes and terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer. The banding pattern of complex microbial communities is simplified by the detection of only terminally fluorescent labeled restriction fragments (Thies, 2007). Analysis of the size, numbers and peak heights of resulting T-RFs are used to estimate diversity in the microbial community. Disadvantages of the T-RFLP method include underestimation of microbial diversity as the number of bands resolved per gel are restricted (<100) and different species can share the same T-RF length (OUT overlap or OUT homoplasy) (Rastogi and Sani, 2011). This technique generates a substantial record of community diversity and is usually well correlated with results from clone libraries (Fierer and Jackson, 2006). The T-RFLP method has been applied in soil (De la Iglesia *et al*., 2006; Osborne *et al*., 2006; Lynch *et al*., 2012), wood (Kirker *et al*., 2012; 2010), and compost (Székely *et al*., 2009) microbial community studies.

1.9.3 Ribosomal Intergenic Spacer Analysis

RISA provides estimates of microbial diversity and community composition without the labour involved as with small-subunit rRNA gene clone library construction. This technique involves PCR amplification of the intergenic spacer region (ISR) between16S and 23S subunit rRNA genes (Fisher and Triplett, 1999). The 16S-23S region contains significant heterogeneity in both length and nucleotide sequence, which is widely used to differentiate bacterial strains and closely related species (Jensen *et al*., 1993). The length heterogeneity of ISR is biased in this method. The PCR product electrophoresed on a polyacrylamide gel is silver stained revealing a banding pattern were each band translates

to an individual microorganism in the community (Fisher and Triplett, 1999). The application of acrylamide gels makes this technique time consuming and troublesome. Automated ribosomal intergenic spacer analysis (ARISA) applies a fluorescence-labeled forward primer with ISR fragments being detected by a laser automatically (Rastogi and Sani, 2011). This automated version enables simultaneous analysis of many samples, although may overestimate microbial intensity and diversity (Fisher and Triplett, 1999). Several studies have implemented this technique in evaluating microbial communities in freshwater (Fisher and Triplett, 1999) and soil environments (Ranjard *et al*., 2001; Lejon *et al*., 2005; Wood *et al*., 2008; Slabbert *et al*., 2010; Khodadad *et al*., 2011).

1.9.4 Denaturing/Temperature Gradient Gel Electrophoresis

As with other culture-independent techniques, DGGE enables the detection of slowly growing, fastidious or uncultivable microorganisms. The reliability of DGGE is very high, as all species present in the community that are over 1% of the total population can be detected (Muyzer and Smalla, 1998). DGGE identifies sequence variants of amplified fragments by the variation in their melting behavior and may be used on both 18S and 16S rDNA (Miller *et al*., 1999). 16S rDNA-based community analysis by DGGE assists in understanding the range of bacterial communities in environmental samples and uncovers much more complex communities than with cultivation (Muyzer *et al*., 1993). The DGGE technique allows for the separation of double-stranded DNA fragments that are identical in length but differ in sequence. During electrophoresis, PCR amplicons migrate towards increasing denaturing concentrations, leading to a partial melting of the DNA helix and to a decrease and eventual immobilization of the DNA product. The TGGE technique applies a temperature gradient as opposed to a denaturing gradient applied in DGGE. A 5'-GC clamped forward primer is included during the PCR step for both techniques. This clamp prevents the two DNA stands from dissociating completely into two single strands during electrophoresis (Miller *et al*., 1999). A banding profile is produced from these methods in which each band represents a microbial species.

Optimum DGGE separation patterns are obtained when short fragments in the range of 200 bp are applied. By excising individual bands, extracting and re-amplifying the DNA,

individual members of the microbial community may be identified (Díez *et al*., 2001, Muyzer *et al*., 1993; Muyzer and Smalla, 1998; Schabereiter-Gurtner *et al*., 2001). However, phylogenetic analyses of sequences obtained directly from DGGE profiles are often challenging due to their short sequence length (Handschur *et al*., 2005). Furthermore, co-migration of different 16S or 18S rDNA sequences, which migrate to the same point in the gel, results in overlapping of DGGE bands which cannot be directly sequenced (Rölleke *et al*., 1999). The problem of identification is compounded by the short DNA fragments, as poor sequence information is obtained by direct sequencing of excised and re-amplified DGGE bands (Ward *et al*., 1990). The appearance of multiple bands in the DGGE of PCR products from pure isolates also needs to be considered when applying this method. This occurrence is explained by the microheterogeneity in the different rRNA-operons present in different species (Muyzer and Smalla, 1998). Fungal diversity in decaying wood has previously been detected using the DGGE of amplified 18S ribosomal DNA (Pennanen *et al*., 2001). DGGE provides a rapid means of examining microbial communities, specifically where the objective is to investigate shifts or variations in community structure (Anderson *et al*., 2003). Its sensitivity and ability to analyse and compare several samples on a single gel and allow a rapid, simultaneous assessment of samples is advantageous. Once the identity of an organism associated with any individual band has been determined, changes in distinct components of a microbial population, due to environmental stresses, can be promptly evaluated (Piñar and Lubitz, 2004).

1.9.5 Polymerase Chain Reaction and Primer Selection

The quality and concentration of extracted nucleic acid is critical for effective PCR amplification of target genomic DNA/RNA. The presence of humic acids co-extracted with nucleic acids from soil or wood can inhibit DNA-modifying enzymes such as *Taq* DNA polymerase (Tebbe and Vahjen, 1993). Extracted DNA is subjected to PCR amplification using primers designed to amplify rRNA genes from particular groups of microorganisms and are known as “universal” primers. This broad amplification of 16S/18S rDNA genes allows the unselective detection of unknown microorganisms in environmental samples (Piñar and Lubitz, 2004). Understanding of the diversity and

ecology of environmental bacterial communities has considerably improved due to variation within 16S rRNA gene sequences of different bacterial species and comparative cataloging since the 1970's (Fox *et al*., 1977). On the contrary, identification of fungi based on sequences of the eukaryotic ribosomal small subunit, the 18S rRNA, is more challenging, with identification commonly limited to genus or family level (Huysmans *et al*., 1983). This is mainly due to the relative lack of variation within 18S rRNA genes between closely related fungal species as a result of the comparatively short period of evolution of the kingdom fungi compared to bacteria. This is compounded by the lack of a comprehensive database of fungal reference sequences (Anderson and Cairney, 2004).

Primer systems existing for fungal community analysis should fulfill the following requirements: (i) specific amplification of representatives from all four major fungal taxonomical groups (Anderson and Cairney, 2004); (ii) amplified fragments should contain enough phylogenetic information to permit distinction of diverse fungal taxa, and (iii) amplification should be highly reproducible and consistent for a large range of environmental samples with varying characteristics (Oros-Sichler, 2006). Anderson *et al*. (2003) suggested some primers to be partial towards certain fungal taxonomic groups, however, the ratio of sequences representing each of the four main fungal phyla, Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota, were similar for each of the primer pairs tested (nu-SSU-1196, nu-SSU-0817 and ITS region), suggesting that primer bias may be less significant than previously thought.

The rRNA gene cluster has been the prime target for the development of PCR primers and despite its limitations, the 18S rRNA gene has been most extensively used for evaluating fungal diversity, exploiting both the conserved and variable regions contained within it (Smit *et al*., 1999; White *et al*., 1990). According to Schabereiter-Gurtner *et al*. (2001), since shorter fragments are necessary for DGGE analysis, 18S rRNA-specific primers lying more upstream to the reverse primer 518R are required. Consequently, the forward primer EF4, constructed for DGGE analysis was combined with primer 518r-GC, producing a 426 bp fragment (Smit *et al*., 1999). In addition, a 316 bp fragment was produced by combining the forward primer NS26, published as a fungal-specific 18S

rDNA primer by Gargas and De Priest (1996) with primer 518R-GC. The ideal primer for 18S amplification would be one that differed between target groups and allowed species-specific differentiation by DGGE or similar techniques. By designing primers, targeting the region from within 18S rDNA to 28S rDNA (Fig. 3), specific taxa such as Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota and Oomycota can be identified (Nikolcheva and Bärlocher, 2004).

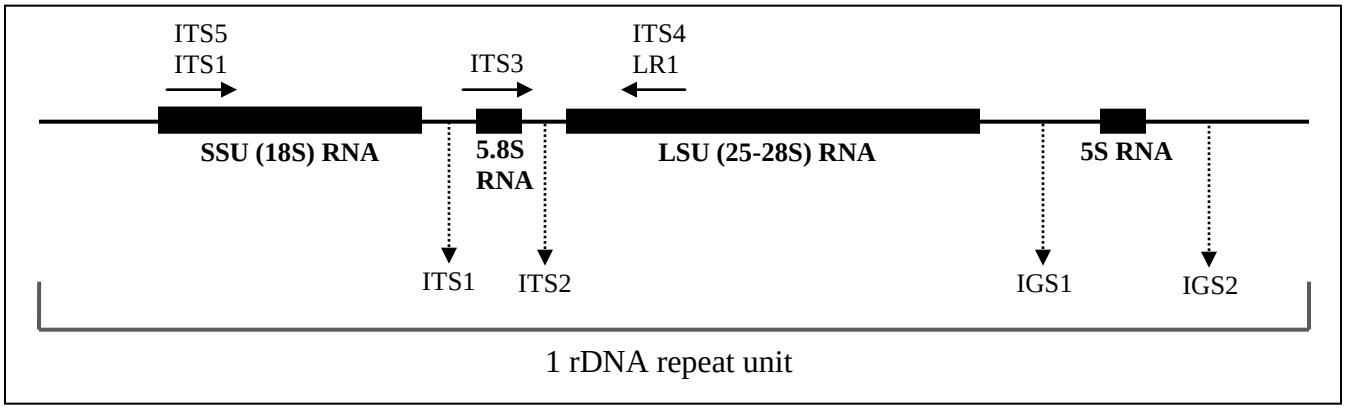

Figure 3: Schematic representation showing the binding sites of the PCR primers along the nuclear gene coding for the SSU rDNA The relative positions of the primers and their direction of extension are indicated by arrows (Vainio and Hantula, 2000; Schabereiter-Gurtner *et al*., 2001; Nikolcheva and Bärlocher, 2004; Oros-Sichler *et al*., 2006).

1.10 PULPING

The purpose of pulping is to remove the cellulose fibres from the rest of the plant material (Breen and Singleton, 1999). Combinations of mechanical and chemical methods are usually used to reduce wood to pulp (Ince, 2004). Chemical pulping involves the addition of chemical reactants and heat energy to soften and dissolve the lignin component in wood. This process is then followed by mechanical refining to split up the fibres. The production of chemical pulps involves digesting the wood chips by utilizing either the sulphate (kraft) or sulphite processes. Generally, the wood chips are cooked with caustic soda to produce black liquor that is removed for the recovery of energy and chemicals (World Bank, 1998).

1.10.1 Mechanical pulping

Mechanical pulping requires wood to be pretreated by the addition of a weak chemical solution or a steaming technique, but is principally reliant on mechanical equipment to reduce wood into fibrous matter by grinding or milling (Ince, 2004). The disadvantage of this method of pulping is that it generates short, weak fibres that still contain the lignin that binds fibres together (Brongers and Mierzwa, 2011). One of the most important standards by which pulping and bleaching methods are measured is the kappa number, which is associated with the pulp yield (Christov *et al*., 1998).

1.10.2 Chemical pulping

Generally the chemical process involves 'cooking' the wood chips with a chemical solution in a heated digester for a period of time followed by refining of the material (Ince, 2004). Chemical pulping is able to disband lignin from the cellulose and hemicellulose fibres (Breen and Singleton, 1999). The pulp produced by this method contains fibres that are clean and undamaged. This type of wood pulp is called woodfree and is used in the manufacturing of all Sappi fine papers (Sappi, 2011).

1.10.2.1 Kraft pulping

The primary chemical pulping process used today is known as the kraft process. In this step the wood chips are cooked in a solution comprised of sodium hydroxide and sodium sulfide. The yield from this process is lower when compared to mechanical pulping since some cellulose is degraded (Breen and Singleton, 1999). Another disadvantage of using chemicals is the obvious threat it poses to the mill workers and the environment (Breen and Singleton, 1999). Interestingly, it appears that the delignification process is influenced more by alterations of residual lignin in wood that has been biotreated than the amount removed for each individual wood component (Vicentim and Ferraz, 2007). Kraft pulp may be used for the production of containerboard, mechanical printing grades, extensible sack kraft and machine glazed kraft (Sappi, 2011).

1.10.2.2 Sulphite pulping

The sulphite process employs a cooking acid composed of free sulphur acid and sulphur acid bound as magnesium bi-sulphite. As the cooking acid penetrates the wood, the lignin is degraded and converted into a water-soluble substance that can be washed out. Sulphite pulp is slightly brown and requires a chlorine-free bleaching step thereafter (Sappi, 2011). The pre-treatment of *E. grandis* with *C. subvermispora* has been shown to facilitate the acid sulphite pulping and bleaching processes (Christov *et al*., 1998).

1.10.3 Parameters of pulp quality

Dissolving pulp can be characterized by various chemical properties such as viscosity, kappa number and carbohydrate content (Elg-Christofferson *et al*., 1999). Control of processes in pulp mill operations are at times complicated as disruptions in pulping conditions, caused by wood supply heterogeneity, can significantly affect pulp bleachability, yield and quality. Although many studies corroborate these facts, their understanding at a molecular level is incomplete, particularly for *Eucalyptus* wood (Colodette *et al*., 2002).

1.10.3.1 Viscosity and Pulp Yield

Pulp yield is vital in reducing the cost of production and enhancing competitiveness in a pulping mill. It is therefore necessary to accurately and appropriately predict the pulp yield (Liu *et al*., 2012). Pulp yield is typically 50-53% for hardwood and 46-49% for softwood (Behin *et al*., 2008). Evaluation of hardwoods has demonstrated that higher cooking liquor alkalinity has the capability of increasing digester throughput without any negative effect on pulp yield and viscosity and with improved bleachability (Rawat and McDonough, 1998). Conversely, it has been shown that pulping with lower alkalinity results in pulps of higher hemicellulose content, which leads to reduced energy requirements during refining (Hanna *et al*., 1998). Barrichelo *et al*. (1983) found that pulp yield is highly correlated with hemicellulose content. A connection between hemicellulose and pulp yield was also established by Wehr (1991) in a study with four lots of *E. grandis*. The author also found that woods with low concentrations of extractives and lignin are related to improved yield. Since bleaching chemicals are more

expensive than cooking chemicals, during the cooking process maximal lignin removal is achieved. However, extensive lignin removal increases cellulose degradation, thus decreasing pulp strength and yield (Behin *et al*., 2008).

Viscosity measurements or other methods are essential parameters for the characterization of pulp. Viscosity is associated with the length of cellulose chains and can be measured in various solvents (Krässig, 1996). The viscosity of pulp signifies the average degree of polymerization of cellulose fibres, therefore indicating relative degradation (decrease in cellulose molecular weight) following the pulping or bleaching process (Tappi, 1996).

1.10.3.2 Kappa number

The lignin content in a chemical pulp is an significant parameter for process control in both pulping and bleaching. One way of assessing the residual lignin content in pulp fibres is by determining the kappa number (Li and Gellerstedt, 1997). The kappa number is the volume (milliliters) of 0.1 *N* potassium permanganate solution consumed by one gram of moisture-free pulp under the conditions specified in this method (TAPPI, 1993). At a given optimum kappa number (e.g. 16-18), hardwood kraft pulps may demonstrate significant variability in pulp yield, bleachability and quality. Many of these variations result from wood variability itself, but others depend upon the pulping processes (Colodette *et al*., 1999) and conditions (Lammi and Svedman, 1999). The relationship between pulp yield and kappa number does not really reflect the relationship between pulp yield and lignin content (Liu *et al*., 2012). Today, the typical kappa number of pulp to be bleached is 14-20 for hardwood and 20-30 for softwood pulp. If the pulp is not bleached, the kappa number after cooking will be much higher, typically 40-100 (Behin *et al*., 2008).

1.10.3.3 Alpha cellulose

The capacity to produce high yield and high purity alpha cellulose pulp is vital in a dissolving pulp mill (Turner, 2001). There is an intense effort focused on the preparation of high-alpha dissolving pulps without unfavorable effects on the pulp. In general,

dissolving pulps reach a limiting α-cellulose content of about 95% (w/w), where the remaining 5% consists of hemicellulose and lignin (Gübitz *et al*., 1998). Near Infra-Red Spectroscopy (NIRS) is used to determine the chemical components of the wood including pulp yield, cellulose, hemicellulose and lignin (Schimleck, 1997).

1.10.3.4 Klason lignin

Lignin (also known as "Klason lignin") is defined as a wood or pulp component insoluble in 72% sulfuric acid (TAPPI, 2002). Lignin removal is a key objective of pulping and bleaching processes. Determination of lignin content in wood and pulps provides information for assessment and application of the processes. Hardness, bleachability and other pulp properties, such as colour, are also associated with the lignin content (TAPPI, 1996).

1.10.3.5 S_{10} and S_{18}

Brightness (S_{10}) and alkali solubility (S_{18}) are essential parameters of dissolving pulp providing information on the bleachability and degradation of cellulose or hemicellulose during pulping and bleaching, respectively. High hemicellulose content (S_{18}, degree of polymerization [DP] of up to 50) and degraded cellulose content (S_{10}-S_{18}, DP of 50-150) affect the degree of swelling of pulp, xanthation reaction and other essential characteristics in the viscose process (Hinck *et al*., 1985).

1.10.4 Sappi

Sappi-Chemical Cellulose is the world's largest manufacturer of Elemental Chlorine Free (ECF) chemical cellulose (dissolving pulp), producing about 800 000 tons per annum. Chemical cellulose is sold to converters for use in a wide range of consumer products such as fashion clothing, mobile phone screens, cellophane wrap, pharmaceutical, beauty and household products. Currently its Chemical Cellulose division supplies customers around the world from the Sappi-Chemical Cellulose Mill in Umkomaas, KwaZulu-Natal (KZN) (http://kzntopbusiness.co.za/site/top-business-sector/Sappi-SAICCOR-(Pty)-Ltd/page/181). Almost all of the pulp produced is exported to countries in Europe, North America, South America and Asia. Approximately 555 000 hectares of land are owned

by Sappi Forests in South Africa. It is estimated that more than 35 million tons of timber occupies up to 380 000 hectares of this land. The dominant type of timber used in Sappi mills is *Eucalyptus* hardwoods as they form 80% of the materials supplied to Sappi. Four different *Eucalyptus* species are planted and harvested for pulp and paper manufacturing viz, *E. grandis*, *E. dunnii*, *E. nitens* and a hybrid of *E. grandis* and *E. nitens*. The KZN and Mpumalanga plantations produce pulpwood and sawlogs, whilst the Usutu Mill in Swaziland produces pulpwood only (www.sappi.com/SappiWeb/About+Sappi/Sappi+Saiccor/Our+company.htm).

There are five 'quality sites' in the KZN region. Quality types 1 and 2 are located in the Zululand area and quality types 2 to 5 in the temperate regions of the Midlands. Site quality is defined by: (i) how fast the trees grow; (ii) tree density; (iii) and the soil quality and weather conditions of the area. The high quality pulp produced by Sappi is used in the textile industry and sold to converters for a wide range of consumer products, such as clothing, cellular phone screens, cellophane wrap for sweets and flowers, pharmaceutical and household products, and makeup such as lipstick (Sappi, 2011). Black liquor, which is a lignin rich waste produced by the pulping process, is used as a biofuel and is fed into the boilers to generate their own electricity. The remaining lignin is supplied to LignoTech (subsidiary of Sappi) and is used as a binding agent, emulsion stabilizer, dust suppressant, etc. (Sappi, 2011).

1.11 GREEN BIOTECHNOLOGY

Immense efforts are being made in developing alternative methods to replace the toxic chemical preservatives currently being used to avoid wood degradation. Numerous investigations are looking towards biological control methods in order to reduce the impact on the environment. The use of toxic chemicals may be eliminated by utilizing biological control methods (Bruce and Highley, 1991; Brown *et al*., 1994; Ejechi, 2003; Verma *et al*., 2007). Ejechi (2003) has reported that the treatment of wood with a combination of *T. viride*, *Proteus* sp. and urea inhibited the biodegradation of wood. In the United States and Europe, a commercially available white mutant of *O. piliferum* called Cartapip 97® is generally applied to softwood chips to prevent sapstain and minimize pitch (de Beer *et al*., 2003). Methods in improving the quality of pulp produced

has lead towards utilizing green biotechnology in the pulping and bleaching processes of paper making (Savitha *et al*., 2009).

Fungi have also been applied in the biological treatment of wood, prior to pulping, for the removal of steroids implicated in pitch deposit formation in chlorine-free pulps (Gutiérrez *et al*., 2001). The most problematic occurrence in paper pulp manufacturing is the development of colloidal pitch, which is caused by lipophilic compounds that form wood resin (Gutiérrez *et al*., 2009). These pitch deposits may be lessened (decrease in resin) by storage of the wood as chips in a wood yard. This is because the chips provide a greater surface area, thus increasing chemical and microbial transformation rates. White-rot basidiomycetes have been reported to cause resin acid degradation, which could be effective in reducing the toxicity of mechanical pulping effluents (Gutiérrez *et al*., 2009). The addition of corn steep liquor may also be applied in order to promote fungal growth, thus reducing the size of inoculum for efficient wood colonization (Akhtar *et al*., 1998). The amount of alkali used in kraft pulping may be reduced by the removal of extractives during biodegradation (Vicentim and Ferraz, 2007).

1.11.1 Biopulping

Biopulping involves the biotreatment of wood with a white-rot fungus and the successive processing of biotreated wood chips by means of mechanical and chemical pulping (Vicentim and Ferraz, 2007). *C. subvermispora* is the most popular microorganism applied as a biopulping agent, as it has great potential for both soft and hardwoods (Christov *et al*., 1998; Breen and Singleton, 1999; Mosai *et al*., 1999; Akhtar *et al*., 2000; Ferraz *et al*., 2008). The use of this fungus as a pretreatment step results in at least 30% reduction of energy utilized during mechanical pulping, and in chemical pulping an increase in pulp yield and reduction in alkali requirements is observed (Akhtar *et al*., 1998; Kang *et al*., 2003). By producing stronger pulp with longer fibres and increased fibrillation, biomechanical pulping may reduce the amount of kraft pulp required to increase pulp strength. Bioreactors of different types are used in biopulping; these include open chip piles, which are only applicable to certain microorganisms in order to get optimal results. The colonization and infiltration of fungal hyphae is vital during the

biotreatment phase and is greatest when the moisture of the wood is between 55-60% (Ferraz *et al*., 2008). The characteristics of a model biopulping strain are: high lipase activity, initiating the least amount of cellulose loss and providing adequate lignin alteration in two weeks or less (Akhtar *et al*., 1997; de Koker *et al*., 2000). The only disadvantage in biopulping is the decrease in brightness of the pulp (Breen and Singleton, 1999).

A comprehensive evaluation of biopulping showed that selected lignin-degrading fungi can be economically grown on wood chips in an outdoor chip pile-based system. Results also demonstrated the great potential of fungal pretreatment of wood chips prior to chemical pulp production. The most prominent benefit of fungal pretreatment is improved effects on cooking, leading to reduced kappa numbers/reduced active alkali charge and/or reduced cooking time after only 1-2 weeks of fungal treatment. Fungal pretreatment also reduces the pitch content in the wood chips and improves the pulp quality in terms of brightness, strength, and bleachability. The bleached biopulps are easier to refine than the reference pulps (Bajpai, 2012). Cartapip 97® may be applied to pulpwood chips to prevent sapstain and minimize pitch (de Beer *et al*., 2003). Sitholé *et al*. (2002) evaluated the effects of Cartapip 97® on aspen chips in South Africa and reported higher strength properties of the pulp compared to untreated chips, after a three week period.

1.11.2 Biobleaching

Bleaching involves the removal of lignin from pulp which ultimately results in white and bright pulp (Dhiman *et al*., 2009). Due to the large amount of chlorine-based chemicals currently being used to bleach pulp, an environmentally friendly alternative would be the application of microbial enzymes to reduce waste and pollution (Bajpai *et al*., 2006). Biobleaching has been implemented by the use of either lignolytic or hemicellulolytic enzymes (Viikari *et al*., 1986; Paice *et al*., 1988). The xylan component of wood may be removed by the application of xylanases, thus improving efficiency of lignin extraction from wood by other pulping and bleaching techniques and reducing the amount of chemicals required to achieve the same degree of brightness and improved physical

properties (Savitha *et al*., 2009; Garg *et al*., 2011). The use of low molecular weight xylanases are particularly more valuable because the smaller enzymes are capable of further invading the fibre structure and are proficient in manipulating the pulp properties (Savitha *et al*., 2009). In addition to this, saprotrophic basidiomycetes may also be applied since they are considered to be proficient decomposers of fixed organic compounds such as lignin and plant cell wall polysaccharides (Baldrian, 2008). Kaur *et al*. (2010) reported the use of specific enzymes in the pretreatment of pulp, with significant increases in pulp quality and properties. In addition, chemical bleaching of treated pulp revealed a 25% reduction in chlorine consumption.

1.12 SCOPE OF THE PRESENT STUDY

The majority of previous studies on wood chip piles have focused on the introduction of bacterial or fungal species onto sterile wood chips to observe changes in the chemical and physical characteristics of the wood (Flannigan and Sagoo, 1977; Ejechi, 2003; Vicentim and Ferraz, 2007; Gelbrich *et al*., 2008). Numerous investigations have also focused on decay basidiomycetes (Adair *et al*., 2002); bacteria (Gelbrich *et al*., 2008; Nilsson *et al*., 2008) or the isolation of specific microbial species from hardwoods or chip piles (Eslyn and Davidson, 1976; De Koker *et al*., 1998; De Koker *et al*., 2000; Roux *et al*., 2004; Beauchamp *et al*., 2006; Kluczek-Turpeinen *et al*., 2007; Grobbelaar *et al*., 2010). Indigenous bacterial and fungal communities are capable of producing a variety of enzymes for the degradation of lignocellulosic materials and the density of their populations vary with changes in the microenvironment and antagonistic effects of other microbes (Hogland and Marques, 2003; Bucher *et al*., 2004; Folman *et al*., 2008). A recent review focused on the effects of microorganisms in large-scale wood piles, however these wood chips were intended for biofuel production (Noll and Jirjis, 2012). The woody biomass utilized in biofuel production is composed of cellulose, lignin and hemicellulose, however for the purposes of dissolving pulp production, high cellulose content is required with minimal or no lignin and hemicellulose (Sannigrahi *et al*., 2010). During the pulping process the use of contaminated or damaged wood chips, in which the cellulose content has been reduced, may become overcooked leading to poor pulp quality. To our knowledge there are no reports on the indigenous microflora inhabiting

commercial wood chip piles intended for dissolving pulp production. It is, therefore important to establish both fungal and bacterial populations in wood chip piles as well as assess the potential for lignocellulolytic production by those microorganisms.

This study seeks to understand the microflora variations in commercial wood chip piles, individual *Eucalyptus* sp. piles and a combination pile during different seasons. Correlations between wood chip quality, chemical and physical properties of the wood species and the microbial variations were also determined.

1.12.1 Hypothesis tested

It is hypothesized that the presence and seasonal variation of microorganisms in wood chips may be correlated to the changes in physical and chemical properties of the wood chips. It is further hypothesized that a specific combination of *Eucalyptus* species may assist in the management of commercial wood chip piles to prevent/reduce harmful degradation of wood components by microorganisms and improve/maintain the quality of dissolving pulp.

1.12.2 Objectives

The following objectives were established to test the above hypothesis: (i) To establish both fungal and bacterial populations and assess whether there is seasonal variation in wood chips: intended for acid bi-sulphite pulping at Sappi-Chemical Cellulose (Umkomaas); of *E. dunnii* and *E. nitens*, of a combination of *E. dunnii* and *E. nitens*, of the above individual wood species and combination exposed to simulated environmental conditions; (ii) To assess physical and chemical properties of wood chips mentioned above; and (iii) To assess whether there is a correlation between the presence of microorganisms and the physical and chemical changes in wood chips.

1.12.3 Experimental design

In the first phase of this study, samples were collected from different areas of the sub piles located in section three, which is situated near reclaimer 340 and 15 in the woodyard at Sappi (Umkomaas). A total of five samples were taken from the north,

south, east, west and middle area within each selected area, in order to obtain a representative sample of the pile. Thirty samples were collected during winter and thirty during summer. In phase two of sampling, the individual wood species were obtained from Sappi (*E. nitens* and *E. dunnii*) for the setup of the winter (16°C and 60% humidity) and summer (25°C and 65% humidity) simulations and control piles. Three kilograms of wood chips were weighed out and placed into three individual piles in a climatic growth chamber. Samples were collected at time zero and after two and four weeks of incubation. Three samples were collected from each of the piles: section 1, 2 and 3 representing the top middle and bottom of the pile, respectively. Fifteen kg of each wood species was transported to Sappi-Chemical Cellulose (Umkomaas) and deposited into enclosures that were constructed. These enclosures allowed for exposure to the natural environment at the woodyard, and served as the Sappi environmental control. An additional 15 kg control piles were set up at University of KwaZulu-Natal (Westville) premises. In the third phase, two of the individual wood species were combined and exposed to simulated conditions. Samples were collected as mentioned above. Wood chips obtained during all three phases of sampling were processed as follows: A portion of each sample was stored as is for microscopy and physical analysis (these analyses were not within the scope of this study). Another portion was milled at the Forestry and Forest Products (FFP) department at CSIR for the identification of microbial species and detection of enzyme activity. The remainder of the chips were sent as is for chemical analyses, and processed accordingly.

1.13 REFERENCES

Adair, S., S. H. Kim, and C. Breuil. 2002. A molecular approach for early monitoring of decay basidiomycetes in wood chips. *FEMS Microbiology Letters* **211:**117 – 122

Akhtar, M., G. M. Scott, R. E. Swaney, and D. F. Shipley. 2000. Biomechanical pulping: a mill-scale evaluation. *Resources, Conservation and Recycling* **28:**241 – 252

Akhtar, M., R. A. Blanchette, G. Myers, and K. Kirk. 1998. An overview of biomechanical pulping research, **p.** 309 – 83. *In*: R. A. Young and M. Akhtar (ed.), Environmentally friendly technologies for the pulp and paper industry. John Wiley and Sons, New York

Akhtar, M., A. Robert, R. A. Blanchette, and T. K. Kirk. 1997. Fungal delignification and biomechanical pulping of wood. *Advances in Biochemical Engineering/Biotechnology* **57:**159 – 195

Anderson, I. C., and J. W. G. Cairney. 2004 Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology* **6:**769 – 779

Anderson, I. C., C. D. Campbell, and J. I. Prosser. 2003. Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environmental Microbiology* **5:**36 – 47

Argyropoulos, D. S., and S. B. Menchem. 1997. Lignin, **p.** 128. *In*: K. Eriksson and E. L. (ed.), Biotechnology in the Pulp and Paper Industry. Springer-Verlag, Berlin

Bajpai, P. 2012. Biopulping, **p.** 67– 92. *In*: Biotechnology for Pulp and Paper Processing. Springer, United States

Bajpai, P., A. Anand, N. Sharma, S. P. Mishra, P. K. Bajpai, and D. Lachenal. 2006. Enzymes improve ECF bleaching of pulp. *BioResources* **1:**34 – 44

Baldrian, P. 2008. Wood-inhabiting ligninolytic basidiomycetes in soils: Ecology and constraints for applicability in bioremediation. *Fungal Ecology* **I:**4 – 12

Barrichelo, L. E. G., J. O. Brito, H. T. Z. Couto, and E. Campinhos Junior. 1983. Densidade básica, teor de holocelulose e rendimento em cellulose de madeiras de *Eucalyptus grandis*. *Silvicultura*, São Paulo, **8:**802 – 808

Beauchamp, C. J., A. Simao-Beaunoir, and C. Beaulieu. 2006. Confirmation of *E. coli* among other thermotolerant coliform bacteria in paper mill effluents, wood chips screening rejects and paper sludges. *Water Research* **40:**2452 – 2462

Béguin, P., and J.-P. Aubert. 1994. The biological degradation of cellulose. *FEMS Microbiology Reviews* **13:**25 – 58

Behin, J., F. Mikaniki, and Z. Fadaei. 2008. Dissolving pulp (alpha-cellulose) from corn stalk by kraft process. *Iranian Journal of Chemical Engineering* **5:**14 – 28

Biely, P. 1985. Microbial xylanolytic systems. *Trends in Biotechnology* **3:**286 – 290

Bisaria, V. S. 1991. Bioprocessing of Agro-residues to glucose and chemicals, **p.** 210 – 213. *In*: A. M. Martin, (ed.), Bioconversion of Waste Material to Industrial Products. Elsevier, London

Blanchette, R. A. 1984. Selective delignification of eastern hemlock by *Ganoderma tsugae*. *Phytopathology* **74:**153 – 160

Blanchette, R. A., T. A. Burnes, M. M. Eerdmans, and M. Akhtar. 1992. Evaluating Isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. *Holzforschung* **46:**109 – 115

Blanchette, R. A., and I. D. Reid. 1986. Ultrastructural aspects of wood delignification by *Phlebia* (*Merulius*) *tremellosus*. *Applied and Environmental Microbiology* **52:**239 – 245

Boland, D. J., M. I. H. Brooker, J. W. Turnball, and D. A. Kleinig. 1980. *Eucalyptus* Seed. Division of Forest Research CSIRO. Canberra, Australia **p.** 191

Boreham, G. R. 2004. Species growth and yield in the warm and cool temperature hardwood zones, part 2: *Eucalyptus macarthurii* and *Eucalyptus nitens* paired plot survey. *Sappi Forests Research Document 20/2004*. Sappi Forests Research, Howick

Breen, A., and F. L. Singleton. 1999. Fungi in lignocellulose breakdown and biopulping. *Current Opinion in Biotechnology* **10:**252 – 258

Bridge, P., and B. Spooner. 2001. Soil fungi: diversity and detection. *Plant and Soil* **212:**147 – 154

Brongers, M. P. H., and A. J. Mierzwa. 2011. *Pulp and Paper*. CC Technologies Laboratories, In., Dublin, Ohio. www.corrosioncost.com (10 September 2011)

Brown, R. A., R. L. Pilling, and D. C. Young. 1994. Preservation of Wood Chips. United States Patent 5342629

Bruce, A., and T. L. Highley. 1991. Control of growth of wood decay *Basidiomycetes* by *Trichoderma* spp. and other potentially anatagonistic fungi. *Forest Products Journal* **41:**63 – 67

Bucher, V. V. C., K. D. Hyde, S. B. Pointing, and C. A. Reddy. 2004. Production of wood decay enzymes, mass loss and lignin solubilization in wood by marine ascomycetes and their anamorphs. *Fungal Diversity* **15:**1 – 14

Campbell, N. A., and J. B. Reece. 2002. Biology. Sixth edition. Benjamin Cummings, San Francisco, CA

Cho, H-B., J-K. Lee, and Y-K. Choi. The genetic diversity analysis of the bacterial community in groundwater by denaturing gradient gel electrophoresis (DGGE). *The Journal of Microbiology* **41:**327 – 334

Christov, L. P., M. Akhtar, and B. A. Prior. 1998. The potential of biosulfite pulping in dissolving pulp production. *Enzyme and Microbial Technology* **23:**70 – 74

Clarke, C. R. E. 2000. Wattle control, Plant Protection Research Institute Handbook No. 3, PPRI, Hilton, KwaZulu-Natal

Clarke, C. R. E., and W. Jones. 1998. Cold tolerant Eucalypt programme breeding manual. Sappi Forests Research, Shaw Research Centre, Howick

Clausen, C. A. 1996. Bacterial associations with decaying wood: a review. *International Biodeterioration & Biodegradation* **37:**101 – 107

Cohen, R., O. Yarden, and Y. Hadar. 2002. Lignocellulose Affects Mn^{2+} Regulation of Peroxidase Transcript Levels in Solid-State Cultures of *Pleurotus ostreatus*. *Applied and Environmental Microbiology* **68:**3156 – 3158

Colodette, J. L., J. L. Gomide, R. Girard, A. Jääskeläinen, and D. S. Argyropoulos. 2002. Influence of pulping conditions on *Eucalyptus* kraft pulp yield, quality, and bleachability. *TAPPI Journal* **1:**14 – 20

Colodette, J. L., J. L. Gomide, D. S. Argyropoulos, Y. A. M. Robles, J. M. Almeida, S. K. Mehlman, and A. G. H. deBrito. 1999. Effect of pulping processes on bleachability with ECF, Z-ECF and TCF bleaching. *Appita Journal* **52:**368 – 374

Coughlan, M. P., M. G., Tuohy, E. X., Filho, J., Puls, M., Claeyssens, M., Vranská, and M. M. Houghes. 1993. Enzymological aspects of microbial hemicellulases with emphasis on fungal systems, **p.** 53–84. *In*: M. P. Coughlan and G. P. Hazlewood, (ed.), Hemicelluloses and hemicellulases. Portland Press, London

Coughlan, M. P., and L. G. Ljungdahl. 1988. Comparative biochemistry of fungal and bacterial cellulolytic enzyme system, **p.** 11 – 30. *In*: J.-P. Aubert, P. Beguin, J. Millet, (ed.), Biochemistry and Genetics of Cellulose Degradation. Academic Press, London

Coutinho, T. A., O. Preisig, J. Mergaert, M. C. Cnockaert, K. –H. Riedel, J. Swings, and M. J. Wingfield. 2002. Bacterial blight and dieback of *Eucalyptus* species, hybrids, and clones in South Africa. *Plant Disease* **86:**20 – 25

Coutinho, T. A., J. Roux, K-H. Riedel, J. Terblanche, and M. J. Wingfield. 2000. First report of bacterial wilt caused by *Ralstonia solanacearum* on *Eucalyptus* in South Africa. *Forest Pathology* **30:**205 – 210

Cowling, E. B., and W. Merrill. 1996. Nitrogen in wood and its role in wood deterioration. *Canadian Journal of Botany* **44:**1539 – 1554

Christakopoulos, P., W. Nerinckx, D. Kekos, B. Macris, and M. Claeyssens. 1996. Purification and characterization of two low molecular weight mass alkaline xylanases from *Fusarium oxysporum* F3. *Journal of Biotechnology* **51:**181 – 189

Crous, P. W., A. J. L. Phillips, and A. P. Baxter. 2000. Phytopathogenic Fungi from South Africa. University of Stellenbosch, Department of Plant Pathology Press, University of Stellenbosch Printers

Crous, P. W., M. J. Wingfield, and S. H. Koch. 1990. New and interesting records of South African fungi. X. New records of *Eucalyptus* leaf fungi. *South African Journal of Botany* **56:**583 – 586

De Beer, Z. W., B. D. Wingfield, and M. J. Wingfield. 2003. The occurrence of *Ophiostoma piliferum*-like fungi on pulpwood chips and other wood sources in South Africa. *South African Journal of Science* **99:**34 – 36

De Koker, T. H., J. Zhao, D. Cullen, and B. J. H. Janse. 1998. Biochemical and molecular characterization of South African strains of *Phanerochaete chrysosporium*. *Mycological Research* **102:**88 – 92

De Koker, T. H., J. Zhao, S. F. Allsop, and B. J. H. Janse. 2000. Isolation and enzymic characterization of South African white-rot fungi. *Mycological Research* **104:**820 – 824

De la Iglesia, R., D. Castro, R. Ginocchio, D. van der Lelie, and B. González. 2006. Factors influencing the composition of bacterial communities found at abandoned copper-tailings dumps. *Journal of Applied Microbiology* **100:**537 – 544

Dhiman, S. S., G. Garg, R. Mahajan, N. Garg, and J. Sharma. 2009. 'Single lay out' and 'mixed lay out' enzymatic processes for bio-bleaching of kraft pulp. *Bioresource Technology* **100:**4736 – 4741

Dianese, J. C., M. C. G. Dristig, and A. P. Cruz. 1990. Susceptibility to wilt associated with *Pseudomonas solanacearum* among six species of *Eucalyptus* growing in equatorial Brazil. *Plant Pathology* **19:**71 – 76

Díez, B., C. Pedrós-Alió, T. L. Marsh, and R. Massana. 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. *Applied and Environmental Microbiology* **67:**2942 – 2951

Duff, S. J. B., and W. D. Murray. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. *Bioresources Technology* **55:**1 – 33

Duong, L. M., R. Jeewon, S. Lumyong, and K. D. Hyde. 2006. DGGE coupled with ribosomal DNA gene phylogenies reveal uncharacterized fungal phylotypes. *Fungal Diversity* **23:**121 – 138

Ejechi, B. O. 2003. Tropical field assessment of combined *Trichoderma viride Proteus* sp. and urea protective treatment of wood against biodeterioration. *International Biodeterioration & Biodegradation* **51:**109 – 114

Elg-Christoferson, K., J. Hauksson, U. Edlund, M. Sjöström, and M. Dolk. 1999. Characterisation of dissolving pulp using designed process variables, NIR and NMR spectroscopy, and multivariate data analysis. *Cellulose* **6:**233 – 249

Eslyn, W. E., and R. W. Davidson. 1976. Some wood-staining fungi from pulp-wood chips. *Memoirs of the New York Botanical Garden* **28:**50 – 57

Fan, L. T., M. M. Gharpuray, and Y.-H. Lee. 1987. Cellulose hydrolysis. *In*: Biotechnology monographs, Springer, berlin, **p.** 57

FAO. Global forest resources assessment 2005 - main report. FAO Forestry Paper, 2005; available online: ftp://ftp.fao.org/docrep/fao/008/A0400E/A0400E00.pdf. Accessed 5 April 2010

Fengxia, L., L. Mei, L. Zhaoxin, B. Xiaomei, Z. Haizhen, and W. Yi. 2008. Purification and characterization of xylanase from *Aspergillus ficuum* AF-98. *Bioresource Technology* **99:**5938 – 5941

Ferraz, A., A. Guerra, R. Mendonça, F. Masarin, M. P. Vicentim, A. Aguiar, and P. C. Pavan. 2008. Technological advances and mechanistic basis for fungal biopulping. *Enzyme and Microbial Technology* **43:**178 – 185

Ferrero, F., C. Lohrer, B. M. Schmidt, M. Noll, and M. Malow. 2009. A mathematical model to predict the heating-up of large scale wood piles. *Journal of Loss Prevention in the Process Industries* **22:**439 – 448

Fierer, N., and R. B. Jackson. 2006. The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America* **103:**626 – 631

Fisher, M. M., and E. W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Applied and Environmental Microbiology* **65:**4630 – 4636

Flannigan, B., and G. S. Sagoo. 1977. Degradation of wood by *Aspergillus fumigatus* isolated from self-heated wood chips. *Mycologia* **69:**514 – 523

Folman, L. B., P. J. Klein Gunnewiek, L. Boddy, and W. de Boer. 2008. Impact of white-rot fungi on numbers and community composition of bacteria colonizing beech wood from forest soil. *FEMS Microbiological Ecology* **63:**181 – 191

Fox, G. E., K. J. Peckman, and C. R. Woese. 1977. Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to prokaryotic systematics. *International Journal of Systemic Bacteriology* **27:**44 – 57

Francescato, V., E. Antonini, and L. Z. Bergomi. 2008. Wood fuels handbook: production, quality requirements and trading. Italian Agriforestry Energy Association, Legnaro, Italy, **p.** 10 – 21

Fuller, W. S. 1985. Chip pile storage – a review of practices to avoid deterioration and economic losses. *Tappi Journal* **68:**48 – 52

Gardner, R. A. W., and T. Swain. 1996. Snow damage to timber plantations in KwaZulu-Natal during July 1996 and subsequent species recommendations. ICFR Bulletin series 08/1996. Institute for Commercial forestry Research, Pietermaritzburg, South Africa

Garg, G., S. S. Dhiman, R. Mahajan, A. Kaur, and J. Sharma. 2011. Bleach-boosting effect of crude xylanase from *Bacillus stearothermophilus* SDX on wheat straw pulp. *New Biotechnology* **28:**58 – 64

Gargas, A., and P. T. De Priest. 1996. A nomenclature for fungal PCR primers with examples from intron-containing SSU rDNA. *Mycologia* **88:**745 – 748

Gelbrich, J., C. Mai, and H. Militz. 2008. Chemical changes in wood degraded by bacteria. *International Biodeterioration & Biodegradation* **61:**24 – 32

Gibbs, R. D. 1958. The Mäule reaction, lignins, and the relationship between woody plants, **p.** 269 – 312. *In*: K. V. Thimann, (ed.), The physiology of forest trees. Ronald Press Company, New York

Gich, F. B., E. Amer, J. B. Figueras, C. A. Abella, M. D. Balaguer, and M. Poch. 2000. Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). *International Microbiology* **3:**103 – 106

Greaves, H. 1971. The Bacterial Factor in Wood Decay. *Wood Science and Technology* **5:**6 – 16

Grobbelaar, J. W., Z. W. de Beer, P. Bloomer, M. J. Wingfield, and B. D. Wingfield. 2010. *Ophiostoma tsotsi* sp. nov., A wound-infesting fungus of hardwood trees in Africa. *Mycopathologia* **169:**413 – 423

Grobbelaar, J. W., Z. W. de Beer, P. Bloomer, M. J. Wingfield, X. Zhou, and B. D. Wingfield. 2011. Discovery of *Ophiostoma tsotsi* on *Eucalyptus* wood chips in China. *Mycoscience* **52:**111 – 118

Gryzenhout, M., B. E. Eisenberg, T. A. Coutinho, B. D. Wingfield, and M. J. Wingfield. 2003. Pathogenicity of *Cryphonectria eucalypti* to *Eucalyptus* clones in South Africa. *Forest Ecology and Management* **176:**427 – 437

Gübitz, G. M., D. W. Stebbing, C. I. Johansson, and J. N. Saddler. 1998. Lignin-hemicellulose complexes restrict enzymatic solubilization of mannan and xylan from dissolving pulp. *Applied Microbiology and Biotechnology* **50:**390 – 395

Gutiérrez, A., J. C. del Río, and A. T. Martínez. 2009. Microbial and enzymatic control of pitch in the pulp and paper industry. *Applied Microbiological Biotechnology* **82:**1005 – 1018

Gutiérrez, A., J. C. del Rio, M. J. Martínez, and A. T. Martínez. 2001. The biotechnological control of pitch in paper pulp manufacturing. *Trends in Biotechnology* **19:**340 – 348

Gutiérrez, A., J. C. del Rio, and F. J. González-Vila. 1998. Variation in the composition of wood extractives from *Eucalyptus globulus* during seasoning. *Journal of Wood Chemistry and Technology* **184:**439 – 446

Häger, B. O. 1986. Preservative Treatment of Wood. *United States Patent* 4597940

Hale, M. D., and R. A. Eaton. 1985. The Ultrastructure of Soft Rot Fungi. I. Fine Hyphae in Wood Cell Walls. *Mycologia* **77:**447 – 463

Handschur, M., G. Pinar, B. Gallist, W. Lubitz, and A. G. Haslberger. 2005. Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. *Food and Chemical Toxicology* **43:**1595 – 1605

Hanna, K. R., and J. J. Fisher. 1998. *International Pulp Bleaching Conference Proceedings*, Tappi Press, Atlanta, Georgia. Book 2, **p.** 323 – 328

Hatakka, A. 2001. Biodegradation of lignin, **p.** 129–80. *In*: M. Hofrichter and A. Steinbüchel, (ed.), Biopolymers, vol. 1, Lignin, humic substances and coal. Wiley-VCH, Weinheim, Germany

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiology Reviews* **13:**125 – 135

Hatfield, R., and W. Vermerris. 2001. Lignin formation in plants. The dilemma of linkage specificity. *Plant Physiology* **126:**1351 – 1357

Herbert, M. A. 2000. Timber Plantation Silviculture, Eucalypt and Wattle species. Forestry Handbook, Southern African Institute of Forestry, Menlo Park. Sec 3: **p.** 85 – 94

Hinck, J. F, R. L. Casebier, and J. K. Hamilton. 1985. Dissolving pulp manufacture, **p.** 213 – 243. *In*: M. G. Kocurek, O.V. Ingruber and P.E. Wong, (ed.), Pulp and Paper Manufacture. Joint Textbook Committee of the Paper Industry, Montreal

Hofrichter, M. 2002. Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology* **30:**454 – 466

Hogland, W., and M. Marques. 2003. Physical, biological and chemical processes during storage and spontaneous combustion of waste fuel. *Resources, Conservation and Recycling* **40:**53 – 69

Huysmans, E., E. Dams, A. Vandenberghe, and R. de Wachter. 1983. The nucleotide sequences of the 5S rRNAs of four mushrooms and their use in studying the phylogenetic position of basidiomycetes among the eukaryotes. *Nucleic Acids Research* **11:**2871 – 2880

Ince, P. J. 2004. Pulping: Fiber Resources, **p.** 877 – 883. *In*: J. Burley, J. Evans and J. A. Youngquist (ed.), Encyclopedia of Forest Sciences, Vol. 2. Elsevier Ltd., Madison, WI, USA

Jeffries, T. W. 1994. Biodegradation of lignin and hemicelluloses, **p.** 233 – 277. *In*: C. Ratledge, (ed.), Biochemistry of Microbial Degradation. Kluwer Academic Publishers, Netherlands

Jensen, M. A., J. A. Webster, and N. Straus. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology* **59:**945 – 952

Ji, N., B. Peng, G. Wang, S. Wang, and X. Peng. 2004. Universal primer PCR with DGGE for rapid detection of bacterial pathogens. *Journal of Microbiological Methods* **57:** 409-413

Jiang, Z., Y. Wei, D. Li, L. Li, P. Chai, and I. Kusakabe. 2006. High-level production, purification and characterization of a thermostable β-mannanase WY34. *Carbohydrate Polymers* **66:**88 – 96

Johnson, K. G., M. C. Silva, C. R. Mackenzie, H. Schneider, and J. D. Fontana. 1989. Microbial degradation of hemicellulosic materials. *Applied Biochemistry and Biotechnology* **20-21:**245 – 258

Kaur, A., R. Mahajan, A. Singh, G. Garg, and J. Sharma. 2010. Application of cellulase-free xylano-pectinolytic enzymes from the same bacterial isolate in biobleaching of kraft pulp. *Bioresource Technology* **101:**9150 – 9155

Kent, A. D., and E. W. Triplett. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annual Reviews Microbiology* **56:**211 – 236

Khodadad, C. L. M., A. R. Zimmerman, S. J. Green, S. Uthandi, and J. S. Foster. 2011. Taxa-specific changes in soil microbial community composition induced by pyrogenic carbon amendments. *Soil Biology & Biochemistry* **43:**8

Kim, Y. S., and A. P. Singh. 2000. Micromorphological characteristics of wood biodegradation in wet environments: a review. *IAWA Journal* **21:**135 – 155

Kinegam, S., S. Tanasupawat, and A. Akaracharanya. 2007. Screening and identification of xylanase-producing bacteria from Thai soils. *Journal of General and Applied Microbiology* **53:**57 – 65

Kirker, G. T., S. V. Diehl, and M. L. Prewitt. 2010. The usual suspects: fingerprinting microbial communities involved in decay of treated Southern Yellow Pine. *Proceedings of the One Hundred Sixth Annual Meeting of the American Wood Protection Association*, May 23-25, Alabama, USA

Kirker, G. T., M. L. Prewitt, T. P. Schultz, and S. V. Diehl. 2012. Community analysis of preservative-treated southern pine (*Pinus* spp.) using terminal restriction fragment length polymorphism (T-RFLP) analysis. Part 1: Fungal field study. *Holzforschung* **66:**521 – 527

Klaassen, R. K. W. M. 2008. Bacterial decay in wooden foundation piles – Patterns and causes: A study of historical pile foundations in the Netherlands. *International Biodeterioration & Biodegradation* **61:**45 – 60

Kluczek-Turpeinen, B., P. Maijala, M. Hofrichter, and A. Hatakka. 2007. Degradation and enzymatic activities of three *Paecilomyces inflatus* strains grown on diverse lignocellulosic substrates. *International Biodeterioration & Biodegradation* **59:** 283 – 291

Krässig, H. A. 1996. Cellulose – structure, accessibility and reactivity, **p.** 216. *In*: M. B. Huglin, (ed.), Polymer Monographs Vol. 11. Gordon and Breach science publishers, Amsterdam

Kuhad, R. C., A. Singh, and K.-E. L. Eriksson. 1997. Microorganisms and enzymes involved in the degradation of plant fiber cell walls, **p.** 47 – 111. *In*: K.-E. L.

Eriksson, (ed.), Biotechnology in the Pulp and Paper Industry. Springer-Verlag, Berlin

Kunz, R., and R. A. W. Gardner. 2001. A review of factors affecting snow damage of commercial forest plantations in South Africa. ICFR Bulletin Series 13/2011. Institute for Commercial forestry Research, Pietermaritzburg, South Africa

Lammi, L., and M. Svedman. 1999. Tailoring pulp quality in the pulping process. *In*: Second ABTCP delignification Seminar. Sáo Paulo, Brazil, September 2-3 http://www.google.co.za/url?sa=t&rct=j&q=lammi%2C%20l.%2C%20and%20m.%20svedman.%201999.%20tailoring%20pulp%20quality%20in%20the%20pulping%20process.%20&source=web&cd=8&cad=rja&ved=0CGcQFjAH&url=http%3A%2F%2Fwww.jstage.jst.go.jp%2Farticle%2Fjtappij%2F59%2F4%2F59_4_483%2F_pdf&ei=x1ZJUaG6J8PRhAe3ooGIAw&usg=AFQjCNHgE1yPGtoGjCPBIRSwDzmpvKINsQ

Lehtikangas, P. 2001. Quality properties of pelletised sawdust, logging residues and bark. *Biomass and Bioenergy* **20:**351 – 360

Lehtikangas, P. 2000. Storage effects on pelletised sawdust, logging residues and bark. *Biomass and Bioenergy* **19:**287 – 293

Lejon, D. P. H., R. Chaussod, J. Ranger, and L. Ranjard. 2005. Microbial community structure and density under different tree species in an acid forest soil (Morvan, France). *Microbial Ecology* **50:**614 – 625

Li, J., and G. Gellerstedt. 1998. On the structural significance of the kappa number measurement. *Nordic Pulp and Paper Research Journal* **13:**153 – 158

Li, J., and G. Gellerstedt. 1997. The contribution to kappa number from hexeneuronic acid groups in pulp xylan. *Carbohydrate Research* **302:**213 – 218

Li, X., H. Koseki, and M. Momota. 2006. Evaluation of danger from fermentation-induced spontaneous ignition of wood chips. *Journal of Hazardous Materials* **A135:**15 – 20

Liu, J., H. Hu, and X. Luo. 2012. Development of a novel empirical model to estimate the kraft pulp yield of fast-growing *Eucalyptus*. *BioResources* **7:**1134 – 1144

Low, C. B., and C. J. A. Shelbourne. 1999. Performance of *Eucalyptus globulus*, *E. maidenii*, *E. nitens* and other eucalypts in Northland and Hawke's Bay at ages 7 and 11 years. *New Zealand Journal of Forestry Science* **29:**274 – 288

Lynch, H. B., K. Y. Epps, T. Fukami, and P. M. Vitousek. 2012. Introduced canopy tree species effect on the soil microbial community in a Montane tropical forest. *Pacific Science* **66:**141 – 150

Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* **66:**506 – 577

Maki, M., K. T. Leung, and W. Qin. 2009. The prospects of cellulose-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences* **5:**500 – 516

Martinez D., L. F. Larrondo, N. Putnam, M. D. S. Gelpke, K. Huang, J. Chapman, K. G. Helfenbein, P. Ramaiya, J. C. Detter, F. Larimer, P. M. Coutinho, B. Henrissat, R. Berka, D. Cullen and D. Rokhsar. 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium strain RP78.* ***Nature Biotechnology*** **22**:695-700

Martin-Laurent, F., L. Philippot, S. Hallet, R. Chaussod, J. C. Germon, G. Soulas, and G. Catroux. 2001. DNA extraction from soils: old bias for new microbial diversity analysis methods. *Applied and Environmental Microbiology* **67:**2354 – 2359

McMurry, J. 2000. Organic Chemistry. 5th edition. Brooks/Cole, CA, USA

Miller, K. M., T. J. Ming, A. D. Schulze, and R. E. Withler. 1999. Denaturing gradient gel electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. *Biotechniques* **27:**1016 – 1018

Miller, R. B. 1999. Wood handbook: wood as an engineering material. Forest Products Laboratory, Madison, WI

Moldes D., M. Díaz, T. Tzanov and T. Vidal. 2008. Comparative study of the efficiency of synt hetic and natural mediators in laccase-assisted bleaching of eucalyptus kraft pulp. *Bioresource Technology* **99**: 7959–7965

Morris, A., and R. Pallett. 2000. Site requirements and species matching: Pines, **p.** 80 – 84. *In*: D. L. Owen, (ed.), South African Forestry Handbook Vol 1. South African Institute of Forestry, Pretoria

Mosai, S., J. F. Wolfaardt, B. A. Prior, and L. P. Christov. 1999. Evaluation of selected whire-rot fungi for biosulfite pulping. *Bioresource Technology* **68:**89 – 93

Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59:**695 – 700

Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* **73:**127 – 141

Muyzer, G. 1999. Genetic fingerprinting of microbial communited – present status and future perspectives. Methods of microbial community analysis. *Proceedings of the 8th International Symposium on Microbial Ecology*. Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Nikolcheva, L. G., and F. Bärlocher. 2004. Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. *Mycological Progress* **3:**41 – 49

Nilsson, T., C. Björdal, and E. Fällman. 2008. Culturing erosion bacteria: Procedures for obtaining purer cultures and pure strains. *International Biodeterioration & Biodegradation* **61:**17 – 23

Noll, M., and R. Jirjis. 2012. Microbial communities in large-scale wood piles and their effects on wood quality and the environment. *Applied Microbiology Biotechnology* **95:**551 – 563

Nurmi, J. 1999. The storage of logging residue for fuel. *Biomass and Bioenergy* **17:**41 – 47

Oakley, A. J., T. Heinrich, C. A. Thompson, and M. C. J. Wilce. 2003. Characterization of a family 11 xylanase from *Bacillus subtillis* B230 used for pulp bleaching. *Acta Crystallographica* **D59:**627 – 636

Osborne, C. A., G. N. Rees, Y. Bernstein, and P. H. Janssen. 2006. New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. *Applied and Environmental Microbiology* **72:**1270 – 1278

Oros-Sichler, M., N. C. M. Gomes, G. Neuber, and K. Smalla. 2006. A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of fungal communities. *Journal of Microbiological Methods* **65:**63 – 75

Paice, M. G., R. Bernier, and L. Jurasek. 1988. Viscosity enhancing bleaching of hardword kraft pulp with xylanase from a cloned gene. *Biotechnological Bioengineering* **32:**235 – 239

Pavlic, D., B. Slippers, T. A. Coutinho, and M. J. Wingfield. 2007. *Botryosphaeriaceae* occurring on native *Syzygium cordatum* in South Africa and their potential threat to *Eucalyptus*. *Plant Pat*hology **56:**624 – 636

Parry, N. J., D. E. Beever, E. Owen, W. Nerinckx, M. Claeyssens, J. V. Beeumen, et al. 2002. Biochemical characterization and mode of action of a thermostable endoglucanase purified from *Thermoascus aurantiacus*. *Archives of Biochemistry and Biophysics* **404:**243 – 253

Pennanen, T., L. Paavolainen, and J. Hantula. 2001. Rapid PCR-based method for direct analysis of fungal communities in complex environmental samples. *Soil Biology & Biochemistry* **33:**697 – 699

Pérez, J., J. Muñoz-Dorado, T. de la Rubia, and J. Martínez. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology* **5:**53 – 63

Piñar, G., and W. Lubitz. 2004. Molecular Techniques: Application to the Analysis of Microbial Communities Colonising Art Works and to the Monitoring of Changes. Case Study: Wall Paintings of the Castle of Herberstein, **2:**421 – 432. *In*: M. Drdácký (ed.), European Research on Cultural Heritage, State-of-the-Art Studies. Academy of Sciences of the Czech Republic, Prague

Potts, B., and H. S. Dungey. 2001. Hybridisation of *Eucalyptus*: Key issues for bredders and geneticists. *Proceedings of Developing the Eucalypt of the Future*. IUFRO Working Party 2.08.03, Valdivia, Chile 8-13 September, Invited paper **p.** 34

Pogue, T. E. 2008. Wood, paper and pulp. Final report commissioned by the Department of Labour for the Sector skills research project. Pretoria: Human Sciences Research Council **p.** 132 – 149

Poutanen, K., M., Tenkanen, H., Korte, and J., Puls. 1991. Accessory enzymes involved in the hydrolysis of xylans. *In*: Enzymes in biomass conversion, Leatham, G. F., and M. E., Himmel (Eds.), Boston. **p.** 427 – 436

Procópio, R. E. L., W. L. Araújo, W. Maccheroni Jr., and J. L. Azevedo. 2009. Characterization of an endophytic bacterial community associated with *Eucalyptus* spp. *Online Journal Genetics and Molecular Research* **8:**1408 – 1422

Ralph, J., J. Marita, S. Ralph, R. Hatfield, F. Lu, R. Ede, J. Peng, S. Quideau, R. Helm, J. Grabber, H. Kim, G. Jimenez-Monteon, Y. Zhang, H. Jung, L. Landucci, J. Mackay, R. Sederoff, C. Chapple, and A. Boudet. 2000. Solution-state NMR of lignins, **p.** 55 **–**108. *In*: D. Argyropoulos and T. Rials (ed.), Advances in Lignocellulosic Characterization. TAPPI Press: Atlanta

Ramos, L. P., C., Breuil, and J. N. Saddler. 1992. Comparison of steam pretreatment of *Eucalyptus*, Aspen, and spruce wood chips and their enzymatic hydrolysis. *Applied Biochemistry and Biotechnology* **34/35:**37 – 48

Ranjard, L., F. Poly, J.-C. Lata, C. Mougel, J. Thioulouse, and S. Nazaret. 2001. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Applied Environmental Microbiology* **67:**4479 – 4487

Ranjard, L., F. Poly, and S. Nazaret. 2000. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. *Research in Microbiology* **151:**167 – 177

Rastogi, G., and R. K. Sani. 2011. Molecular techniques to assess microbial community structure, function, and dynamics in the environment, **p.** 516. *In*: Ahmad et al. (ed.), Microbes and Microbial Technology: Agricultural and Environmental Applications. Springer Science+Business Media, LLC

Rawat, N., and T. J. McDonough. 1998. *Tappi Pulping Conference Proceedings*, **p.**883 – 891

Risna, R. A., and Suhirman. 2002. Ligninolytic enzyme production by *Polyporaceae* from Lombok, Indonesia. *Fungal Diversity* **9:**123 – 134

Rockwood, D. L., A. W. Rudie, S. A. Ralph, J. Y. Zhu, and J. E. Winandy. 2008. Energy product options for *Eucalyptus* species grown as short rotation woody crops. *International Journal of Molecular Sciences* **9:**1361 – 1378

Rogers, G. M., and A. A. W. Baecker. 1991. *Clostridium xylanolyticum* sp. nov., an anaerobic xylanolytic bacterium from decayed *Pinus patula* wood chips. *International Journal of Systematic Bacteriology* **41:**140 – 143

Rölleke, S., C. Gurtner, U. Drewello, U. Drewello, R. Drewello, W. Lubitz, and R. Weissmann. 1999. Analysis of bacterial communities on historical glass by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Journal of Microbiological Methods* **36:**107 – 114

Roux, J., M. van Wyk, H. Hatting, and M. J. Wingfield. 2004. *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* **53:**414 – 421

Roux, J., M. J. Wingfield, J-P. Bouillet, B. D. Wingfield, and A. C. Alfenas. 2000. A serious new wilt disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30:**175 – 184

Ruiz-Dueñas, F. J., and A. T. Martínez. 2009. Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. *Microbial Biotechnology* **2:**164 – 177

Sannigrahi, P., A. J. Ragauskas, and G.A. Tuskan. 2010. Poplar as a feedstock for biofuels: a review of compositional characteristics. *Biofuels, Bioproducts & Biorefining* **4:**209 – 226

Sappi ©. 2011. Accessed: June 2010
http://www.sappi.com/regions/sa/SappiSouthernAfrica/Paper%20and%20Paper%20Packaging/Pages/default.aspx
http://kzntopbusiness.co.za/site/top-business-sector/Sappi-SAICCOR-(Pty)-Ltd/page/181
www.sappi.com/SappiWeb/About+Sappi/Sappi+Saiccor/Our+company.htm

Sappi-Chemical Cellulose. 2007. The Paper Making Process: From wood to coated paper. Sappi Europe SA, Accessed: June 2010

http://www.google.co.za/url?sa=t&rct=j&q=the%20paper%20making%20process%3A%20from%20wood%20to%20coated%20paper.%20sappi%20europe%20sa&source=web&cd=1&cad=rja&ved=0CC0QFjAA&url=http%3A%2F%2Fwww.na.sappi.com%2Fdocuments%2F10165%2F13508%2FThe%2BPapermaking%2BProcess.pdf&ei=YldJUZ7uDtO5hAep_oHwBg&usg=AFQjCNEAVE4i5vYUqXZZw8CtGBYFITjf3g

Savitha, S., S. Sadhasivam, and K. Swaminathan. 2009. Modification of paper properties by the pretreatment of wastepaper pulp with *Graphium putredinis*, *Trichoderma harzianum* and fusant xylanases. *Bioresource Technology* **100:**883 – 889

Schabereiter-Gurtner, C., G. Piñar, W. Lubitz, and S. Rölleke. 2001. Analysis of fungal communities on historical church window glass by denaturing gradient gel electrophoresis and phylogenetic 18S rDNA sequence analysis. *Journal of Microbiological Methods* **47:**345 – 354

Schimleck, L. R., P. J. Wright, A. J. Michell, and A. F. A.Wallis. 1997. Near-infrared spectra and chemical composition of *Eucalyptus globulus* and *E. nitens* plantation woods. *Appita Journal* **504:**40 – 45

Schönau, A. P. G., and R. A. W. Gardner. 1991. Eucalypts for colder areas in Southern Africa, **p.** 467 – 479. *In*: A. P. G. Schönau, (ed.), Proceedings of IUFRO symposium Intensive Forestry: "The Role of Eucalypts". Durban, South Africa

Shin, C. S, J. P. Lee, J. S. Lee, and S. C. Park. 2000. Enzyme production of *Trichoderma reesii* rut C-30 on various lignocellulosic substrates. *Applied Biochemistry and Biotechnology* **84-84:**237 – 245

Singh, S., P. Reddy, J. Haarhoff, P. Biely, B. Janse, B. Pillay, D. Pillay, and B. A. Prior. 2000. Relatedness of *Thermomyces lanuginosus* strains producing a thermostable xylanase. *Journal of Biotechnology* **81:**119 – 128

Sithole, B., M-J. Rocheleau, R. Berlyn, C. Heitner, and L. Allen. 2002. Is fungal treatment of Aspen chips beneficial for CTMP? Technical Association of the Pulp and Paper Industry of Southern Africa, African Pulp and Paper Week, International Convention Centre, Durban, 8 – 11 October

Sjöström, E. 1993. Wood Chemistry, Fundamentals and Applications. Second edition. San Diego: Academic Press, **p.** 292

Slabbert, E., C. J. van Heerden, and K. Jacobs. 2011. Optimization of automated ribosomal intergenic spacer analysis for the estimation of microbial diversity in fynbos soil. *South African Journal of Science* **106:**329

Slippers, B., G. Fourie, P. W. Crous, T. A. Coutinho, B. D. Wingfield, A. J. Carnegie, and M. J. Wingfield. 2004. Speciation and distribution of *Botryosphaeria* spp. on native and introduced *Eucalyptus* trees in Australia and South Africa. *Studies in Mycology* **50:**343 – 358

Smit, E., P. Leeflang, and K. Wernars. 1997. Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. *FEMS Microbiology Ecology* **23:**249 – 261

Smit, E., P. Leeflang, B. Glandorf, J. D. Van Elsas, and K. Wernars. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied Environmental Microbiology* **65:**2614 – 2621

Smith, A. H., E. A. Pinkard, G. C. Hunter, M. J. Wingfield, and C. L. Mohammed. 2006. Anatomical variation and defence response of juvenile *Eucalyptus nitens* leaves to Mycosphaerella leaf disease. *Australasian Plant Pathology* **35:**725 – 731

Smith, H., M. J. Wingfield, and O. Petrini. 1996. *Botryosphaeria dothidea* endophytic in *Eucalyptus grandis* and *Eucalyptus nitens* in South Africa. *Forest Ecology and Management* **89:**189 – 195

Srebotnik, E., K. A. Jensen, and K. E. Hammel. 1994. Fungal degradation of recalcitrant nonphenolic lignin structures without lignin peroxidase. *Proceedings of the National Academy of Sciences of the United States of America* **91:**12794 – 12797

Srebotnik, E., and K. Messner. 1994. A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white-rot fungi. *Applied and Environmental Microbiology* **60:**1383 – 1386

Subramaniyan, S., and P. Prema. 2003. Biotechnology of microbial xylanases: Enzymology, molecular biology and application. *Critical Reviews in Biotechnology* **22:**33 – 64

Sun, Y., and J. Cheng. 2002. Hydrolysis of lignocellulosic material for ethanol production: a review. *Bioresource Technology* **83:**1 – 11

Székely, A. J., R. Sipos, B. Berta, B. Vajna, C. Hajdú, and K. Márialigeti. 2009. DGGE and T-RFLP analysis of bacterial succession during mushroom compost production and sequence-aided T-RFLP profile of mature compost. *Microbial Ecology* **57:**522 – 533

Tanaka, H., K. Koike, S. Itakura, and A. Enoki. 2009. Degradation of wood and enzyme production by *Ceriporiopsis subvermispora*. *Enzyme and Microbial Technology* **45:**384 – 390

Tansey, M. R. 1971. Isolation of thermophilic fungi from self-heated, industrial wood chip piles. *Mycologia* **63:**537 – 547

TAPPI. 2002. Test Methods T222 om-02: Pentosans in wood and pulp. Tappi Press, Atlanta, USA

TAPPI. 1996. Test Methods T-204-OM-88: Solvent extractives; T-222-OM-88: Acid insoluble lignin of wood and pulp; T249 cm-85: Carbohydrate anaylsis; T235-OM60: Alkali solubility of pulp at 25°C; T203 om-94: Viscosity of pulp (capillary viscometer method). Tappi Test Methods 1996-1997, Tappi Press, Atlanta, USA

TAPPI. 1993. Test Methods T 236 cm-85: Kappa Number. Tappi Press, Atlanta, USA

Tebbe, C. C., and W. Vahjen. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and yeast. *Applied Environmental Microbiology* **59:**2657 – 2665

Temp, U., C. Eggert, and K. L. Eriksson. 1998. A small-scale method for screening of lignin-degrading microorganisms. *Applied and Environmental Microbiology* **64:**1548 – 1549

Thies, J. E. 2007. Soil microbial community analysis using terminal restriction fragment length polymorphisms. *Soil Science Society of America Journal* **71:**579 – 591

Truman, R. 1974. Die-back of *Eucalyptus citriodora* caused by *Xanthomonas eucalypti* sp. *Phytopathology* **64:**143 – 144

Turnbull, J. W. 2000. Economic and social importance of eucalyptus, **p**. 1 – 9. *In*: P. J. Keane, G. A. Kile, F. D. Podger and B. N. Brown (ed.), Diseases and pathogens of *Eucalyptus*. CSIRO Publishing, Australia

Turner, P. 2001. Strategic and tactical options for managing the quality and value of eucalypt plantation resources. FFP, CSIR. IUFRO Conference "Developing the Eucalypt of the future", Valdivia, Chile, 10-15 September.

Uraiuj, C., C. Khanongnoch, and S. Lumyong. 2003. Ligninolytic enzymes from tropical endophytic *Xylariaceae*. *Fungal Diver*sity **13:**209 – 219

Vainio, E. J., and J. Hantula. 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research* **104:**927 – 936

Valášková, V., and P. Baldrian. 2006. Degradation of cellulose and hemicellulose by the brown rot fungus *Piptoporus betulinus* – production of extracellular enzymes and characterization of the major cellulases. *Microbiology* **152:**3613 – 3622

Van Staden, V., B. F. N. Erasmus, J. Roux, M. J. Wingfield, and A. S. van Jaarsveld. 2004. Modelling the spatial distribution of two important South African plantation forestry pathogens. *Forest Ecology and Management* **187:**61 – 73

Verma, M., S. K. Brar, R. D. Tyagi, R. Y. Surampalli, and J. R. Valéro. 2007. Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. *Biochemical Engineering Journal* **37:**1 – 20

Verryn, S. D. 2000. *Eucalyptus* hybrid breeding in South Africa. "Hybrid Breeding and Genetics of Forest Trees" Proceedings of QFRU/CRC-SPF Symposium, 9-14 April 2000. **p.** 191 – 199. Noosa, Queensland, Australia. Department of Primary Industries, Brisbane

Vicentim, M. P., and A. Ferraz. 2007. Enzyme production and chemical alterations of *Eucalyptus grandis* wood during biodegradation by *Ceriporiopsis subvermispora* in cultures supplemented with Mn^{2+}, corn steep liquor and glucose. *Enzyme and Microbial Technology* **40:**645 – 652

Viikari, L., M. Ranua, A. Kantelinen, J. Sundquist, and M. Linko. 1986. Bleaching with enzymes. *In*: Proceedings of the third international conference on biotechnology pulp and paper industry, Stockholm, 16-19 June, **p.** 67 – 69

Viljoen, A., M. J. Wingfield, and P. W. Crous. 1992. Fungal Pathogens in *Pinus* and *Eucalyptus* Seedling Nurseries in South Africa: A Review. *South African Forestry Journal* **161:**45 – 51

Ward, D. M., R. Weller, and M. M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345:**63 – 65

Wardlaw, T. J., G. A. Kile, and J. C. Dianese. 2000. Diseases of eucalypts associated with viruses, phytoplasmas, bacteria and nematodes. ***In***: P. J. Keane, G. A. Kile, F. D. Podger, and B. N. Brown, (ed.), Diseases and pathogens of eucalypts. CSIRO Publishing, Collingwood, Australia

Wehr, T. R. 1991. Variações nas características de Eucalyptus grandis W. Hill ex-Maiden e suas influências na qualidade de cavacos em cozimentos Kraft. Dissertação (Mestrado em Ciências Florestais) – Escola superior de Agricultura "Luiz de Queiroz", Piracicaba 84 f.

White, T. J., T. Burns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, **p**. 315-322. ***In***: M. A. Innes, D. H. Gelfand, J. J. Sninsky and T. J. White, (ed.), PCR Protocols. Academic Press, San Diego, CA

Winandy, J. E. 1994. Wood Properties, **p.** 549 – 561. *In*: C. J. Arntzen and E. M. Ritter, (ed.), Encyclopedia of Agricultural Science. Academic Press, Orlando, Florida

Wingfield, M. J., P. W. Crous, and T. A. Couthinho. 1997. A serious new canker disease of *Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. *Mycopathologia* **136:**139 – 145

Wong, K. K. Y., L. U. L. Tan, and J. N. Saddler. 1988. Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. *Microbiological Reviews* **52:**305 – 317

Wood, S. A., A. Rueckert, D. A. Cowan, and S. C. Cary. 2008. Sources of edaphic cyanobacterial diversity in the dry valleys of Eastern Antarctica. *The International Society for Microbial Ecology Journal* **2:**308 – 320

World Bank. 1998. "Pollution Prevention and Abatement: Pulp and Paper Mills." Project Guidelines: Industry Sector Guidelines. http://books.google.co.za/books?id=S7mj6Laj_q8C&pg=PA400&lpg=PA400&dq=World+Bank.+1998.+%E2%80%9CPollution+Prevention+and+Abatement:+Pulp+and+Paper+Mills.%E2%80%9D+Project+Guidelines:+Industry+Sector+Guidelines.&sour

ce=bl&ots=h6lqQ8yU78&sig=AJrnyGUBhgWPoL6OFdQQ4mDrjks&hl=en&sa=X&ei=qldJUf6hN8OBhAe97oGICQ&ved=0CCsQ6AEwAA

Zak, J. C., and S. Visser. 1996. An appraisal of soil fungal biodiversity: the crossroads between taxonomic and functional biodiversity. *Biodiversity and Conservation* **5:**169 – 183

Zhang, Y-H. P., M. E. Himmel, and J. R. Mielenz. 2006. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances* **24:**452 – 481

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Method Optimization for DGGE Analysis of Microflora from Industrial Wood Chips Intended for Pulping

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Eucalyptus*, pine and wattle are the predominant exotic wood species used in the South African pulp and paper industry. On entering the mill, wood is chipped and stored in piles where it becomes vulnerable to microbial degradation and spontaneous combustion. The PCR-based Denaturing Gradient Gel Electrophoresis technique was optimized for the detection of microbial diversity – both bacterial and fungal - in commercial wood chips. Wood chips were collected from an industrial wood yard and milled to different specifications. A total of four primer sets with GC clamps were tested in nested PCR for DGGE analysis. 16S and 18S rRNA genes were amplified using 338F-GC/518R; 933F-GC/1387R (bacteria) and NS26/518R-GC; EF4F/518R-GC (fungi), respectively. Several gel gradients were examined to determine optimal separation of bacterial (40/60%,35/50%,30/60%) & fungal (35/50%,20/45%,25/50%) PCR-DGGE products. Comparison of the DGGE profiles revealed greater diversity in the milled wood chips amplified using primer sets; 338F-GC/518R (16S) and NS26/518R-GC (18S) with gradients of 30/60% (16S) and 25/50% (18S). After optimization, the standardized protocol was tested against five samples to assess its applicability to wood yard samples. 16S and 18S DGGE profiles were generated and amplicons excised from gels, re-amplified, sequenced and the microorganism from which the DNA originated was determined. Predominant genera were *Klebsiella* spp. (×3), *Bacillus* spp. (×2), *Pantoea* spp. (×2), *Pseudomonas* spp. (×2) and *Paecilomyces* spp. (×2). Using the PCR-DGGE method eighteen (18) bacterial and twelve (12) fungal species were identified, compared to the ten (10) bacterial and nine (9) fungal isolates which were identified using the culturing technique and standard 16S and 18S rRNA gene sequence analysis.

Keywords: Wood chips, *Eucalyptus*, PCR-DGGE, bacteria and fungi.

INTRODUCTION

Eucalyptus species are the predominant hardwood commercial plantations for the production of pulp and paper [1]. Within a few days following harvesting, the wood is debarked, chipped at the mill and stored in piles and thus remains wet and may hold over 50% of their weight as water [2]. The availability of water to microbes initiates the growth of bacteria and promotes fermentation [3]. Storage of wood chips in piles leads to redistribution of the moisture resulting in a wet outer surface and drier inner part [4,5] and may influence microbial succession within the chip piles based on moisture and temperature levels within the piles [6]. Favourable conditions for bacteria are provided by the release of heat by the respiring parenchyma cells in the wood chips.

Erosion and tunneling bacteria are common wood-

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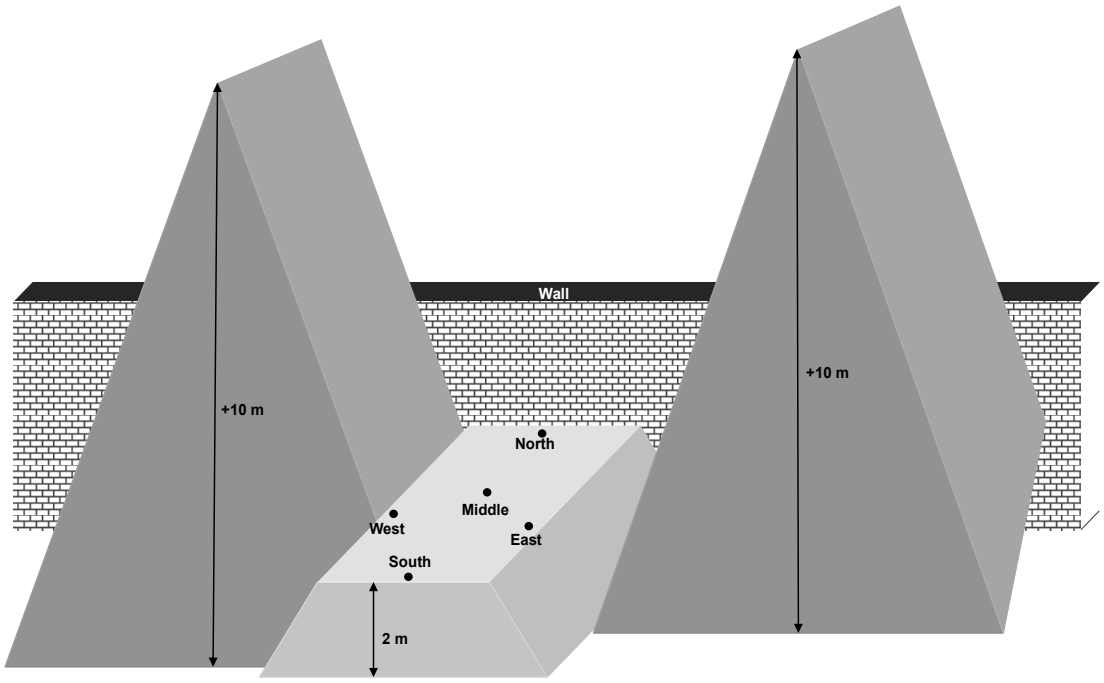

Figure 1. Schematic layout of the wood chip piles at a pulping mill and sampling points (1st July 2010).

inhabiting microorganisms and initial colonizers of wood [7]. Subsequent colonization by fungi is in response to the abundant wood extractives produced by bacterial degradation [8]. The presence of thermophilic and thermotolerant bacteria is dominant in wood chip piles as temperatures reach up to 45-50°C, thus preventing the growth of basidiomycetes [7]. The sources of these microorganisms are:

(i) Different sections of the tree (leaves, bark, needles).
(ii) Colonization of the inner wood by tunneling bacteria and wood-rotting fungi
(iii) Transportation into wood chip piles by wind and rain
(iv) Microbial migration from the soil [5].

During the pulping process, if microbially degraded or physically and chemically damaged wood chips are used they may become overcooked, leading to poor pulp quality. While physical and chemical properties of wood chips have been extensively analyzed, there are limited reports of microbial community within chip piles [5]. It is therefore of interest to establish both fungal and bacterial populations in freshly chipped hardwoods and monitor changes in populations over time to prevent the production of low quality pulp. Previous studies have focused either on bacteria [9-11], fungi [12-15] or the isolation of a particular microorganism (often pathogenic) from wood [16-18]. Many of these studies relied on traditional culturing techniques to study microflora.

Molecular methods relying on DNA or RNA extracted directly from environmental samples such as Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE), Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) are increasingly being used, particularly in analyzing the community structure of microorganisms from different environments, eg. soil [19-22], compost [6,23], wastewater [24-26], food [27-30] and decaying chip piles intended for fuel [13,14]. Several reports focused on understanding the microbial effects on storage of woody biomass intended for biofuel production, as well as composting [6,23,31].

A recent review highlighted the critical factors influencing storage of woody biomass intended for biofuel production but also stated that most reports on microbial communities from wood chips and logs can be regarded as case studies rather than statistical analyses [5]. The lack of data on wood chips for pulping emphasizes the need for this study. The first step in acquiring this data for *Eucalyptus* spp. and examining correlations between microflora, seasons and the chemical and physical properties of wood chips and the final dissolving pulp quality, requires optimization and standardization of the PCR-DGGE technique so that shifts in populations could be attributed to wood species and season and not variation in techniques. This paper demonstrates for the first time the optimization of PCR-DGGE and preliminary application of this standardized protocol to assess its applicability in analyzing the spectrum of microbial species (fungal and bacterial) present in hardwood chips

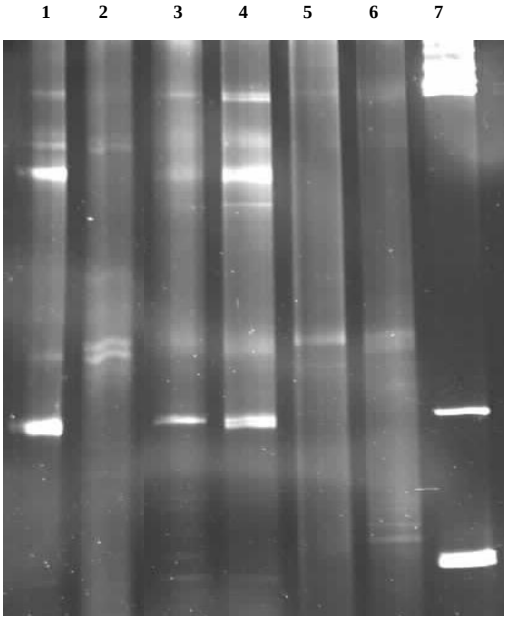

Figure 2. DGGE analysis of 16S GC-PCR products amplified from DNA extracted from the sample collected in the south area (S5) of the wood chip pile. Lanes 1 – 7: un-milled S5 (338f-GC and 518r), un-milled S5 (933f-GC and 1387r), milled S5 (338f-CG and 518r), fine milled S5 (338f-CG and 518r), fine milled S5 (933f-GC and 1387r), milled S5 (933f-GC and 1387r), 100 bp marker

intended for pulping.

MATERIALS AND METHODS

Sample Collection and Preparation

Samples were collected from five different areas/points of a commercial wood chip pile located in an open wood yard at a pulping mill in Umkomaas, on the south coast of South Africa. Samples were collected from a pile that was largely sheltered by two larger piles located on either side (Figure. 1). The pile height was approximately 2 m on the day of sampling. Five samples were collected from different areas (S1=north, S2=middle, S3=east, S4=west and S5=south) from the bottom section of the pile (0.5m below surface level).

The middle and north areas are located closest to a separating wall which provides some shelter from the elements, whilst the remaining areas were mostly exposed. Samples were collected using latex gloves and sterile zip-lock bags (22 x 34 cm) and transported back to the laboratory and temporarily stored (~5 days) at 4°C until the samples could be milled and stored at -20°C. In accordance with TAPPI T 257, air-dried wood chips were ground in a Wiley type mill to coarse sawdust. The saw dust was then passed through a 0.40 mm (40 mesh) screen. Two milled samples were thus obtained >40 mm and finer material <40 mm [32].

Isolation of Bacterial and Fungal Cultures

Five grams of wood chips were thoroughly washed by vortexing with five millilitres of phosphate buffer (pH 8.0) for 5 min. The washings were serially diluted and spread onto nutrient agar (Merck, South Africa) incubated at 37°C for 36 hours (bacteria) and potato dextrose agar (PDA) (Merck, South Africa) incubated at 30°C for 4 to 6 days (fungi). Colonies were selected from the spread plates based on size, shape, pigmentation, margin, consistency and elevation and purified on appropriate agar plates.

DNA Extraction Methods

Genomic DNA was extracted from 0.2 g milled and un-milled chips using the Soil DNA Extraction Kit (Zymo Research, United Sates). A modification was necessary, however, as the milled wood chips absorbed the lysis buffer which had to be increased to 1200 µl per extraction. Genomic DNA was isolated from the pure bacterial and fungal isolates using the ZR Fungal/Bacterial DNA Kit (Zymo Research, United States), as per manufacturer's instructions.

PCR Reactions

Ribosomal genes were amplified from microbial genomic DNA from milled chips, un-milled chips and purified cultures. Universal primer sets for 16S and 18S rRNA were used and amplification conditions are listed in Table 1. Amplification reactions (50 µl) contained 1.25 mm $MgCl_2$, 0.125 µM forward and reverse primers, 0.2 mm dNTPs, 0.25 U SuperTherm *Taq* DNA polymerase (Southern Cross Biotech, South Africa), and approximately 20-200 ng of template DNA (measured with a Nano Drop 1000 Spectrophotometer, Thermo Scientific, USA). The volume of DNA was maintained constant in order to establish a standardised method to monitor community changes over time and season in a later study. PCR was performed using the Gene Amp PCR System 9700 (Applied Biosystems, United States).

The amplicons were analyzed by electrophoresis on 1% agarose (SeaKem, United States) gels in 1×Tris-Acetate EDTA running buffer at 90 V for 45 min. After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide and visualized using the Chemi-Genius 2 BioImaging System (Syngene, United States).

Following PCR, the amplicons were sequenced (Inqaba Biotech, South Africa), sequences edited and entered in the BLAST algorithm [33] for identification of microorganisms. Upon confirmation of 16S and 18S amplicons, products were purified using a Gene JET™ PCR purification kit (Fermentas, Lithuania) and re-

Table 1. Primers and PCR reaction conditions for amplification of 16S and 18S ribosomal genes.

Primer name	Nucleotide sequence (5'-3')	Amplicon length (bp)	Techniques	Reaction Conditions	Reference
63F	CAGGCCTAACACATGCAAGTC	1300	PCR	Initial denaturation: 95°C, 5' 30 Cycles: Denaturation: 95°C, 1' Annealing: 55°C, 1' Extension: 72°C, 1.5' Final Extension: 72°C, 5'	[24,49]
1387R	GGCGGWGTGTACAAGGC				
ITS5F	GGAAGTAAAAGTCGTAACAAGG	600	PCR	Initial denaturation: 95°C, 2' 25 Cycles: Denaturation: 95°C, 30'' Annealing: 53°C, 45'' Extension: 72°C, 1' Final Extension: 72°C, 8'	[39,50]
ITS4R	CCTCCGCTTATTGATATGCTTAAG				
338F* 518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	237	PCR-DGGE	Initial denaturation: 94°C, 5'	[30]
933F*	GCACAAGCGGTGGAGCATGTGG	500	PCR-DGGE	30 Cycles: Denaturation: 95°C, 1' Annealing: 53°C, 1' Extension: 72°C, 2' Final Extension: 72°C, 5'	[28]
1387R	GCCCGGGAACGTATTCACCG				
NS26F 518R*	CTGCCCTATCAACTTTCGA ATTACCGCGGCTGCTGG	316	PCR-DGGE	Initial denaturation: 94°C, 5'	[15]
EF4F	GGAAGGGGTGTATTTATTAG	426	PCR-DGGE	30 Cycles: Denaturation: 95°C, 1' Annealing: 55°C, 1' Extension: 72°C, 1' Final Extension: 72°C, 5'	[15]
518R*	ATTACCGCGGCTGCTGG				

*GC-clamp added to the 5´ end of the primer (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´).

amplified in a touchdown thermal profile program using nested PCR and primers with GC-clamps (Table 1). The composition of the reaction mixtures were the same as that used for the first PCR.

DGGE

DGGE was performed using the D-Code Universal Mutation Detection System (BioRad, United States), modified from Muyzer *et al*. [34]. PCR samples were loaded onto vertical perpendicular polyacrylamide gels (6% acrylamidebisacrylamide (37.5:1) in 1× TAE buffer prepared using 30 and 60% denaturant (100% denaturant corresponds to 7 M urea and 40% formamide).

The following gradients were tested to determine optimal separation of bacterial PCR-DGGE products; 40/60%, 35/50% and 30/60%; while 35/50%, 20/45% and 25/50% gradients were used for bacterial and fungal amplification products, respectively.

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, United States). Bacterial and fungal DNA ladders for DGGE were constructed. They consisted of known microbial species which were isolated, cultured and identified together with bands that were excised, re-amplified and sequenced from other DGGE gels.

RESULTS

DGGE Analysis and Identification of Bacteria and Fungi

Three parameters were optimized for DGGE analysis; gradient choice, primer and sample processing. Optimal gradients for resolution of bacterial and fungal amplicons were 30/60% and 25/50%, respectively. DGGE profiles with greatest variety and visual clarity were produced using primer sets 338F-GC/518R and NS26/518R-GC. A greater variety of species were observed for bacteria (Figure. 2) and fungi (not shown) from the milled chips compared to the fine-milled and un-milled chip samples.

Bacterial and fungal DGGE community profiles of the

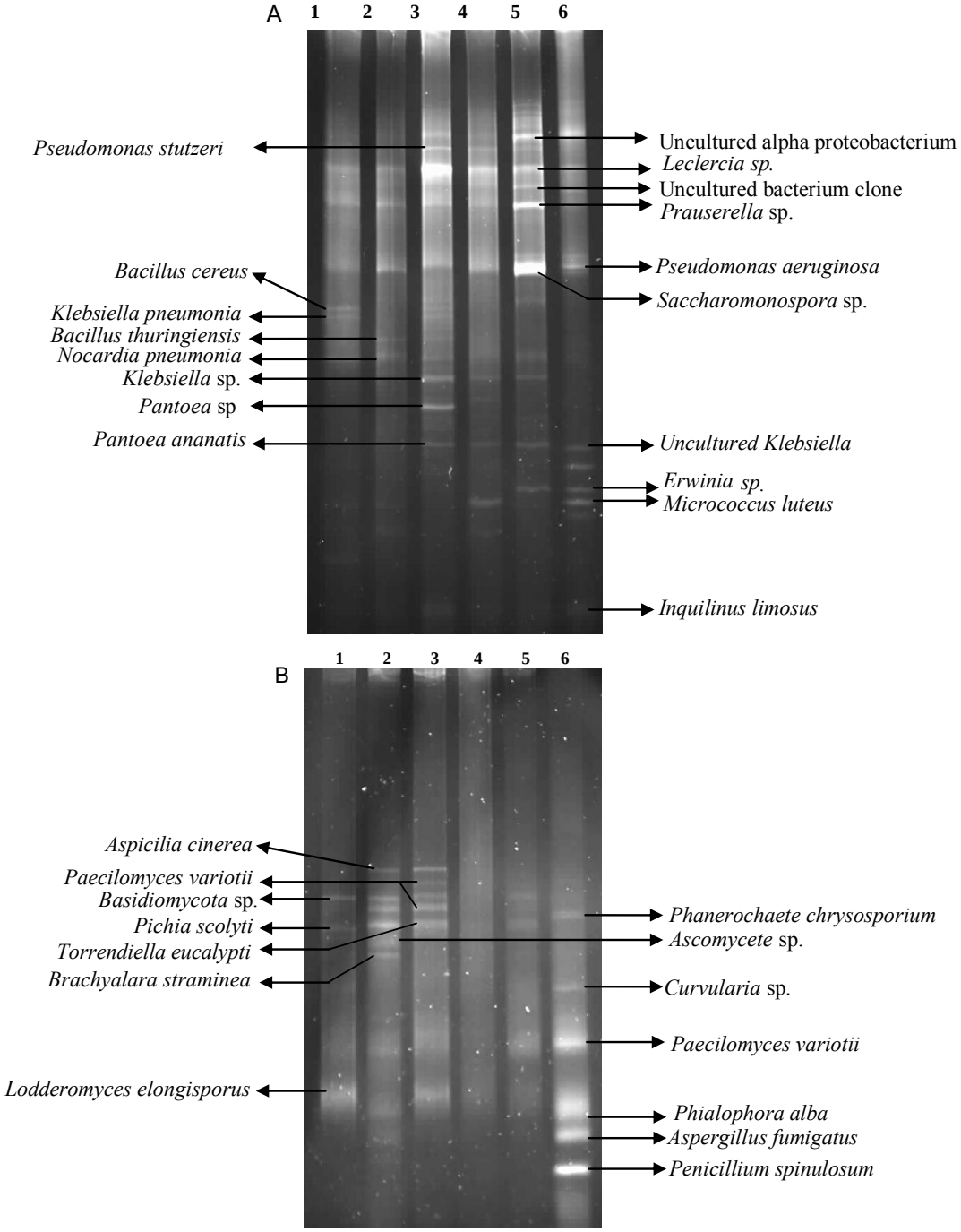

Figure 3. DGGE profiles of 16S and 18S amplicons from DNA extracted from different areas of a wood chip pile. (A) DGGE analysis of 16S GC-PCR products. Primer 338F-GC and 518R: lane 1 – 6; sample S1 (north), S2 (middle), S3 (east), S4 (west), S5 (south), marker **(B)** DGGE analysis of 18S GC-PCR products. Primer NS26 and 518R-GC: lane 1 – 6; sample S1, S2, S3, S4, S5, marker.

different areas sampled are shown in Figure 3 and the identities summarized in Tables 2 and 3. DGGE profiles clearly indicate a diversity of bacterial and fungal strains.

The varying intensity of the bands was an indication of varying population densities/abundance of the species in different sampling areas (Figure. 3). Twenty six (26) and fourteen (14) distinct amplicons were visualized for bacteria and fungi, respectively, using the optimized DGGE method. At least eight bands (representing *Leclercia* spp., *Prauserella* spp., *Pseudomonas stutzeri*,

Table 2. Bacteria identified by sequencing of 16S rRNA amplicons of cultured pure isolates and excised bands from PCR-DGGE gels.

Pure Isolates	Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Accession Number
B1	*Pseudomonas aeruginosa*	Proteobacteria	JX465662.1	100	JX945659
B2	*Pseudomonas aeruginosa*	Proteobacteria	AY946287.1	99	JX945660
B4	*Bacillus firmus* strain	Firmicutes	HQ285922	99	JX945657
B5	*Micrococcus luteus*	Actinobacteria	FN393774.1	99	JX945661
B6	*Bacillus* spp.	Firmicutes	GU905015	97	JX945662
B7	*Inquilinus limosus*	Proteobacteria	AY043375.1	99	JX945663
B9	*Pantoea* spp.	Proteobacteria	HM008959.1	99	JX945664
B10	*Klebsiella* spp.	Proteobacteria	GU797267.1	98	JX945665
B12	*Bacillus ginsengihumi*	Firmicutes	AB245378.1	99	JX945658
B14	*Streptomyces costaricanus*	Actinobacteria	AB249939.1	99	JX945666
B15	*Pseudomonas aeruginosa*	Proteobacteria	AY631240.1	99	JX945667
B16	*Cellulosimicrobium cellulans*	Actinobacteria	GQ503328.1	98	JX945668

DGGE Bands	Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Gaps	Accession Number
1	*Pseudomonas stutzeri*	Proteobacteria	JQ963329.1	99	0/152	KC020158
2	*Leclercia* spp.	Proteobacteria	JX174253.1	97	1/148	KC020159
3	*Klebsiella pneumoniae*	Proteobacteria	JX457349.1	98	1/143	KC020160
4	*Bacillus thuringiensis*	Firmicutes	HQ432813.1	99	1/152	KC020161
5	*Nocardia pneumonia*	Actinobacteria	JF973479.1	93	3/84	*
6	*Klebsiella* spp.	Proteobacteria	AY880196.1	93	4/149	KC020162
7	*Pantoea* spp.	Proteobacteria	AY659872.1	96	0/158	KC020163
8	*Pantoea ananatis*	Proteobacteria	HQ683996.1	95	1/153	KC020164
9	Uncultured alpha proteobacterium	Proteobacteria	EF370684.1	92	2/128	*
10	*Bacillus cereus*	Firmicutes	FR695425.1	97	0/147	*
11	Uncultured bacterium clone	No rank	JF772766.1	94	1/149	*
12	*Prauserella* spp.	Actinobacteria	GU292546.1	93	1/86	*
13	*Pseudomonas aeruginosa*	Proteobacteria	JX514417.1	92	3/132	*
14	*Saccharomonospora* spp.	Actinobacteria	JX244129.1	98	0/66	KC020165
15	Uncultured *Klebsiella* spp.	Proteobacteria	HM053738.1	97	0/150	KC020166

Table 2. Cont.

16	*Erwinia* sp.	Proteobacteria	FM161470.1	97	3/154	KC020167
17	*Micrococcus luteus*	Actinobacteria	FN393774.1	99	1/133	*
18	*Inquilinus limosus*	Proteobacteria	AY043373.1	93	1/127	*

*Accession numbers could not be assigned to excised bands that were less than 150 bp after sequencing.

uncultured *Klebsiella* spp., *Klebsiella* spp. and *Saccharomonospora* spp.) were common in the five samples. *Klebsiella pneumoniae* was found only in the north (S1) and east (S3), *Bacillus thuringiensis* in the north (S1), middle (S2) and east (S3), *Inquilinus limosus* in the east (S3), west (S4) and south (S5), and *Pantoea* spp. in the east (S3) only (Figure. 3A).

The DGGE profile for fungi showed greater variability (Figure. 3B). The greatest variability with the appearance and disappearance of bands was evident for samples from the east (S3) and middle (S2).
Torrendiella eucalypti, *Paecilomyces variotii*, *Basidiomycota* spp. and *Lodderomyces* spp. appeared as bright bands. *Basidiomycota* sp. was present in samples from the north (S1), middle (S2), east (S3) and less abundant in the south (S5) but absent in the west (S4) area. Similarly, *T. eucalypti* were detected in the middle (S2) and east (S3) samples, but faint in the south (S5) sample. It was not possible to sequence nine bands as they were too faint and/or co-migrated. The west (S4) sample did not display any bands; this may be due to insufficient amounts of eukaryotic DNA extracted from this sample.

Ten bacterial and nine fungal species were isolated using the traditional method of culturing and identified using 16S/18S rRNA sequencing. The following species were identified: three *Bacillus* spp., three different *Pseudomonas aeruginosa* isolates, *Inquilinus* spp., *Micrococcus* spp., *Pantoea* spp., *Klebsiella*, *Streptomyces* spp., *Cellulosimicrobium* spp. (Table 2), two *Paecilomyces* spp., two *Aspergillus fumigatus* isolates, *Phanerochaete* spp., *Penicillium* spp., *Phialophora* spp., *Geosmithia* sp., *Acremonium* spp. and *Curvularia* spp. (Table 3). *Bacillus* spp. (33%) and *Aspergillus fumigatus* (29%) were the predominant bacterial and fungal species, respectively.

DISCUSSION

The aim of this investigation was to optimize the PCR-DGGE method to obtain a standardized method to assess the microbial community present in hardwood chips intended for pulping. This was achieved by testing different parameters such as sample specifications (milled), primers (16S-338f-GC/518r; 18S-NS26f/518r-GC) and gradients of DGGE gels (16S-30/60%, 18S-25/50%).

Several DNA isolation techniques were attempted. Initially genomic DNA was extracted directly from the wood chips using the supernatant from the wood chip washings and manual methods of extraction for bacteria (modified from La Montagne et al. [35]), fungi (modified from Miller et al. [29]) and a combination of bacteria/fungi (modified from Zhou et al. [34]). DNA was quantified, however when PCR was conducted, no bands were observed on a 1% agarose gel. Several adjustments to optimize extraction were made to protocols; however, this did not have any effect on PCR amplification although satisfactory amounts of genomic DNA were obtained. It was suspected that the presence of plant phenols in the samples inhibited the PCR reaction, therefore a commercial kit comprising purification columns was ultimately used.

Other studies have reported optimal gradients of 18/58% and 45/60% for fungi [13,14], and 20/60% for bacteria [37]. Various 18S primer sets have been applied in other DGGE analysis studies, namely; NS1-GC/NS2+10 (566 bp) [38], NS1/FR1-GC (1647 bp) [37,38], FR1-GC/FF700 (700 bp) and FR1-GC/FF1100 (1100 bp) [39].

Bacterial community studies applying the DGGE technique used similar primer sets such as; 968F-GC/1401R (433 bp) [19]. Optimum DGGE separation patterns were reported when short fragments in the range of 200 bp were applied to the gel [15,34-41]. This was the basis for the primer selection in this study, as the 16S and 18S primers generated 237 bp and 316 bp amplicons, respectively. This selection therefore, significantly influenced the determination of the gradients applied and optimal separation. Eighteen and 12 bacterial and fungal species, respectively, were identified by sequencing of DGGE bands. Some species remained unidentified due to poor visibility and close proximity of bands leading to underreporting of the full microbial spectrum.

These challenges were also encountered by other authors [19,21,22]. Sample overloading and variation of DGGE gradients may resolve this issue. Another drawback to this technique

Table 3. Fungi identified by sequencing of 18S rRNA amplicons of cultured pure isolates and excised bands from PCR-DGGE gels.

Pure Isolates	Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Accession Number
F1	*Paecilomyces* spp.	Ascomycota	AB217858.1	99	JX945646
F2	*Aspergillus fumigatus*	Ascomycota	GU566217.1	100	JX945647
F3	*Phanerochaete chrysosporium*	Basidiomycota	AF475147.1	100	JX945648
F4	*Paecilomyces formosus*	Ascomycota	GU968673.1	99	JX945649
F5	*Paecilomyces formosus*	Ascomycota	GU968664.1	99	JX945650
F6	*Geosmithia arqillacea*	Ascomycota	GU165722.1	99	JX945651
F7	*Penicillium verruculosum*	Ascomycota	HM469420.1	99	JX945652
F8	*Acremonium implicatum*	Ascomycota	FN706553.1	99	JX945653
F9	*Aspergillus fumigatus*	Ascomycota	GU566217.1	100	JX945654
F10	*Phialophora alba*	Ascomycota	HM116755.1	99	JX945655
F12	*Curvularia* spp.	Ascomycota	HQ631061.1	100	JX945656
DGGE Bands	**Species**	**Phyla Affiliation**	**Best Match database (Gene Bank Accession No.)**	**Similarity (%)**	**Gaps**	**Accession Number**
1	*Aspicilia cinerea*	Ascomycota	DQ986735.1	97	2/236	KC020149
2	*Paecilomyces variotii*	Ascomycota	AB023948.1	99	1/236	KC020150
3	*Basidiomycota* spp.	Basidiomycota	HQ696102.1	97	3/235	KC020151
4	*Pichia scolyti*	Ascomycota	FJ153138.1	98	2/228	KC020152
5	*Torrendiella eucalypti*	Ascomycota	DQ195811.1	94	2/235	KC020153
6	*Brachyalara straminea*	Ascomycota	HQ609482.1	99	2/229	KC020154
7	*Paecilomyces variotii*	Ascomycota	JN256017.1	94	1/208	KC020155
8	*Phialophora alba*	Ascomycota	HM116755.1	99	1/234	*
9	*Aspergillus fumigatus*	Ascomycota	GU992275.1	100	0/238	*
10	*Penicillium spinulosum*	Ascomycota	GU566247.1	99	2/232	*
11	*Lodderomyces elongisporus*	Ascomycota	EF120591.1	98	1/236	KC020156
12	*Ascomycete* spp.	Ascomycota	EU484181.1	99	2/234	KC020157

*Accession numbers could not be assigned to excised bands that were less than 150 bp after sequencing.

however, is that a simple relationship of one band representing one genus/species is not always probable as a single point mutation may sometimes result in two bands. This was evident in the DGGE profile of the pure fungal and bacterial isolates. The micro heterogeneity in the different rRNA-operons present in different species may be responsible for this [34]. Since DGGE detects most single-base substitutions, this would explain the variety of *Klebsiella* spp. that was observed at different migration points in the gel [29].The majority of fungal bands with inferred

identities are known to be either thermotolerant or thermophilic, therefore, it is expected that these isolates would be found towards the middle and back of the pile which was sheltered from cross winds, creating an environment which retains heat generated by microorganisms. Eleven ascomycetes and one basidiomycete were identified from the excised bands.

The majority of endophytic fungi which dominate healthy tissue of almost all wood species are ascomycetes [5]. A number of ascomycetes (soft-rot fungi) are capable of growing at higher levels of temperature, moisture and pH than are tolerated by wood-decaying basidiomycetes [42]. In a study by Rajala *et al*. basidiomycetes dominated decaying spruce logs; however, in slightly decayed logs ascomycetes formed the majority of the inhabiting community [13].

Thus, the dominance of ascomycetes may be attributed to the state of the wood. The universal primer set used (ITS5F/ITS4R) may have also been inadequate in detecting basidiomycetes due to lack of primer binding to DNA isolated from this group of microorganisms. Adair et al utilized primer set ITS1F/NL2R which they reported as specific for the identification of basidiomycetes [12]. ITS5F and ITS1F have binding sites in close proximity to each other whereas NL2 primer set was selected as it covered a larger area of 18S rDNA gene (ITS 1 and 2 region, 5.8S and part of the small ribosome subunit). Limitations such as primer bias and specificity have previously been reported for primer sets [43].

The DGGE technique provides an insight to the relative abundance of microorganisms within a sample as well as variations in populations from different sections of a chip pile. One of the drawbacks to this technique is that less abundant species represented by very faint bands are difficult to excise and sequence. However, by overloading the gels with high concentrations of amplified DNA, bands may become more visible and available for excision and sequencing. Also bands may migrate very close together and make excision difficult. Optimising gradients for regions not well-separated will allow better separation. These two strategies will ensure that a greater variety and number of microorganisms may be identified compared to the culturing technique.

Culturing techniques allowed the isolation of 10 bacterial and nine fungal species (Tables 2 and 3). Based on morphological differences, more isolates were selected for identification, however, sequencing data revealed a few of them to be identical to either *A. fumigatus* or *P. spinulosum*. The microbial genera identified in this study have been reported in other studies on hard and softwood chips in piles and compost, including *Paecilomyces* spp., *Phialophora* spp., *Penicillium* spp., *Curvularia* spp., *Streptomyces* spp., *Bacillus* spp., *Pseudomonas* spp. and *Micrococcus* spp. [42,44,45].

Basic culture media and standard temperatures were used to isolate bacterial and fungal species. If the aim were to isolate as many microbes as possible, then it is recommended that a range of enrichment media and incubation at various conditions (temperature, pH, aeration) be applied [44-47]. For enrichment and isolation of thermophilic species Emerson YpSs Agar (Difco) or 2% malt agar and incubation in damp chambers may be used [45]. Four of the fungal isolates were identified as *Aspergillus fumigatus*. This is expected as *A. fumigatus* is one of the most common fungi isolated from wood chip piles [27,46,48]. Most isolates of *A. fumigatus* show some shade of green, but some also exhibit buff-coloured colonies [46]. This type of variation explained the number of *A. fumigatus* colonies selected for purification and identification.

CONCLUSION

DGGE enabled identification of a greater number of isolates, making it a favourable culture-independent method compared to the basic culture-dependent technique tested. Our results suggest that the optimized PCR-DGGE parameters developed in this study will be suitable for establishing and monitoring changes in microbial communities within wood chip piles in the follow-up study. This paper lays the groundwork for microbial community studies of industrial scale hardwood chip piles intended for dissolving pulp production.

Analysis of lignocellulolytic enzyme production, wood and pulp chemistry, relationships between the natural microflora present and their combined effects on the final pulp yield can assist in determining the potential effect of microflora on dissolving pulp quality.

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REFERENCES

[1] FAO. Global forest resources assessment - main report. FAO Forestry Paper.. available online: ftp://ftp.fao.org/docrep/fao/008/A0400E/A0400E00.pdf. ISBN: 92-5-105481-9, 2005

[2] Brown RA, Pilling RL, Young DC. Preservation of wood chips. United States Patent. 1994; 5342629.

[3] Li X, Koseki H, Momota M. Evaluation of danger from fermentation-induced spontaneous ignition of wood chips. J Hazard Mater. 2006; A135: 15-20.

[4] Bedane AH, Afzal MT, Sokhansanj S. Simulation of temperature and moisture changes during storage of woody biomass owing to weather variability. Biomass Bioen., 2011; 35: 3147-3151.

[5] Noll M, Jirjis R. Microbial communities in large-scale wood piles and their effects on wood quality and the environment. Appl Microbiol Biotechnol., 2012; 95: 551-563.

[6] Novinscak A, DeCoste NJ, Surette C, Filion M. Characterization of bacterial and fungal communities in composted biosolids over a 2 year period using denaturing gradient gel electrophoresis. Can. J. Microbiol., 2009; 55: 375-387.

[7] Clausen CA. Bacterial associations with decaying wood: A Review. Int. Biodeterior. Biodegrad., 1996; 37: 101-107.

[8] Fuller WS. Chip pile storage – a review of practices to avoid deterioration and economic losses. Tappi J., 1985; 68: 48-52.

[9] Gelbrich J, Mai C, Militz H. Chemical changes in wood degraded by bacteria. Int. Biodeterior. Biodegrad., 2008; 61: 24-32.

[10] Kallioinen A, Vaari A, Rättö M, Konn J, Siika-aho M, Viikari L. Effects of bacterial treatments on wood extractives, J. Biotechnol., 2003; 103:67-76.

[11] Nilsson T, Björdal C, Fällman E. Culturing erosion bacteria: Procedures for obtaining purer cultures and pure strains. Int. Biodeterior .Biodegrad., 2008; 61:17-23.

[12] Adair S, Kim SH, Breuil C. A molecular approach for early monitoring of decay basidiomycetes in wood chips. FEMS Microbiol. Lett., 2002; 211: 117-122.

[13] Rajala T, Peltoniemi M, Hantula J, Mäkipää R, Pennanen T. RNA reveals a succession of active fungi during the decay of Norway spruce logs. Fungal Ecol., 2011; 4:437-448.

[14] Rajala T, Peltoniemi M, Pennanen T, Mäkipää R. Relationship between wood-inhabiting fungi determined by molecular analysis (denaturing gradient gel electrophoresis) and quality of decaying logs. Can. J. Res., 2010; 40:2384-2397.

[15] Schabereiter-Gurtner C, Piñar G, Lubitz W, Rölleke S. Analysis of fungal communities on historical church window glass by denaturing gradient gel electrophoresis and phylogenetic 18S rDNA sequences analysis. J. Microbiol. Methods, 2001; 47:345-354.

[16] Grobbelaar JW, de Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD. Ophiostoma tsotsi spp. nov., a wound-infesting fungus of hardwood trees in Africa. Mycopathol., 2010; 169:413-423.

[17] Kluczek-Turpeinen B, Maijala P, Hofrichter M, Hatakka A. Degradation and enzymatic activities of three Paecilomyces inflatus strains grown on diverse lignocellulosic substrates. Int. Biodeterior. Biodegrad., 2007; 59:283-291.

[18] Roux J, van Wyk M, Hatting H, Wingfield MJ. Ceratocystis species infecting stem wounds on Eucalyptus grandis in South Africa. Plant Path, 2004; 53:414-421.

[19] Gelsomino A, Keijzer-Wolters AC, Cacco G, van Elsas JD. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. J Microbial Methods, 1999; 38:1-15.

[20] Hoshino YT, Matsumoto N. Changes in fungal community structure in bulk soil and spinach rhizosphere soil after chemical fumigation as revealed by 18S rDNA PCR-DGGE. Soil Science and Plant Nutrition, 2007; 53:40-55.

[21] Maarit-Niemi R, Heiskanen I, Wallenius K, Lindstrom K. Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. J Microbiol Methods, 2001; 45:155-165.

[22] Xue D, Yao HY, Ge DY, Huang CY. Soil microbial community structure in diverse land use systems: a comparative study using biological, DGGE and PLFA analyses. Pedosphere, 2008; 18:653-663.

[23] Adams JDW, Frostick LE. Analysis of bacterial activity, biomass and diversity during windrow composting. Waste Management, 2009; 29: 598-605.

[24] Moura A, Tacão M, Henriques I, Dias J, Ferreira P, Correia A. Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR-DGGE analysis. Microbial Res, 2009; 164:560-569.

[25] Liu C, Yang JL, Wu G, Zhang S, Li ZX, Guo JB. Estimation of dominant microbial population sizes in the anaerobic granular sludge of a full-scale UASB treating streptomycin wastewater by PCR-DGGE. World J Microbial Biotechnology, 2010; 26:375-379.

[26] Chen H, Lin Y, Fanjiang J, Fan C. Microbial community and treatment ability investigation in AOAO process for the optoelectronic wastewater treatment using PCR-DGGE biotechnology. Biodegraded, 2012; doi 10.1007/s10532-012-9579-0.

[27] Greaves H. Microbiological aspects of wood chip storage in tropical environments. Aust J Biological Science, 1975; 28:315-322.

[28] Ji N, Peng B, Wang G, Wang S, Peng X. Universal primer PCR with DGGE for rapid detection of bacterial pathogens. J Microbial Methods, 2004; 57:409-413.

[29] Miller KM, Ming TJ, Schulze AD, Withler RE. Denaturing gradient gel electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. Bio techniques, 1999; 27:1016-1020.

[30] Handschur M, Piñar G, Gallist B, Lubitz W, Haslberger AG. Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. Food Chem Toxicol, 2005; 43:1595-1605.

[31] Ashraf R, Shahid F, Ali TA. Association of fungi, bacteria and actinomyces with different composts. Pak J Bot, 2007; 39:2141-2151.

[32] TAPPI Test Methods test method number: T 257 cm-12, 2012

[33] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic Local Alignment Search Tool. J Molecular Biology, 1990; 215:403-410.

[34] Muyzer G, Smalla K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek, 1998; 73:127-141.

[35] LaMontagne MG, Michel FC, Holden PA, Reddy CA. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. J Microbial Methods, 2002; 49:255-264.

[36] Zhou J, Bruns MA, Tiedje JM. DNA recovery from soils of diverse composition. Appl Environ Microbial, 1996; 62:316-322.

[37] Li P, Wang X, Yuan X, Wang X, Cao Y, Cui Z. Screening of a composite microbial system and its characteristics of wheat straw degradation. Agric Science in China, 2011; 10:1586-1594.

[38] Kowalchuk GA, Gerards S, Woldendorp JW. Detection and characterization of fungal infections of Ammophila arenia (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. Appl Environ Microbial, 1997; 63:3858-3865.

[39] Vainio EJ, Hantula J. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol Res, 2000; 104:927-936.

[40] Pennanen T, Paavolainen L, Hantula J. Rapid PCR-based method for the direct analysis of fungal communities in complex environmental samples. Soil Biological Biochemistry, 2001; 33:697-699.

[41] Muyzer G, De Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbial, 1993; 59:695-700.

[42] Lindgren RM, Eslyn WE. Biological deterioration of pulpwood and pulp chips during storage. TAPPI, 1961; 44:419-429.

[43] Anderson IC, Cairney JWG. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ Microbial, 2004; 6:769-779.

[44] de Koker TH, Zhao J, Allsop SF, Janse BJH. Isolation and enzymic characterisation of South African white-rot fungi. Mycol Res. 2000; 104:820-824.

[45] Eslyn WE, Davidson RW. Some wood-staining fungi from pulpwood chips. Memoirs of the New York Botanical Garden, 1976; 28:50-57.

[46] Flannigan B, Sagoo GS. Degradation of wood by Aspergillus fumigatus isolated from self-heated wood chips. Mycologia, 1977; 69:514-523.

[47] Shields JK. Microflora of eastern Canadian wood chip piles. Mycologia, 1969; 61:1165-1168.

[48] Tansey MR. Isolation of thermophilic fungi from self-heated, industrial wood chip piles. Mycologia, 1971; 63:537-547.

[49] Marchesi JR, Sato T, Weightman AJ, Fry JC, Hiom SJ. Design and evaluation of useful bacterial-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbial, 1998; 64:795-799.

[50] White TJ, Burns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innes et al. (eds) PCR Protocols, Academic Press, San Diego, CA, 1990; pp 315-322.

DGGE Analysis of Bacteria and Fungi in Commercial Pulping Wood Chip Piles

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Abstract

A cross-sectional study of microflora in wood chip piles from a commercial dissolving pulp mill was conducted in summer and winter. Investigation into microbial diversity was achieved using an optimized PCR-based Denaturing Gradient Gel Electrophoresis (DGGE) technique. Bacterial and fungal strains were identified after sequencing of 16S and 18S rRNA DGGE amplicons. Chemical characteristics of the wood chips were evaluated by conducting extractive analyses using HPLC. DGGE profiles and sequence data indicated a range of bacterial and fungal strains which varied in intensity based on area sampled and season. Seventy nine (45 in summer and 34 in winter) and 29 (20 in summer and 9 in winter) distinct amplicons representing bacteria and fungi, respectively, were visualized. Predominant genera in summer were *Pantoea rodasii*, *Inquilinus limosus*, *Streptococcus* sp., *Klebsiella* spp., *Diversispora* sp., *Boletaceae* sp., *Scutellospora* sp., and *Ophiostoma bicolour* and in winter were *Leuconostoc palmae*, *Streptococcus* sp., *Bacillus* spp., *Diversispora* sp., *Boletaceae* sp., and *Bullera* sp. Cellulose, hot water extractive and acid insoluble levels correlated significantly with season. Lower cellulose levels in summer correlated significantly with high microbial loads and the predominance of *Bacillus* spp., suggesting that in warm humid environments storage should not exceed 1-2 weeks. Decreased hot water level in winter could not be correlated to microbial activity but were correlated to increased exposure of those samples to environmental factors.

1. Introduction

In South Africa *Eucalyptus* spp. form the predominant species in the pulping industry due to their short growth cycle, chemical and physical properties (Pogue 2008). Debarked trees are transported to the pulping plant and processed into uniform wood chips, to facilitate storage and cooking, and stored in outdoor piles (Sappi-Chemical Cellulose, 2007). Storage

of wood as chips is essential as it is more economical to handle chips rather than logs and logs will need to be reduced to chips for efficient pulping (Quillin 1994). However, the disadvantage of this is the development of higher temperatures inside the wood chip piles which are less dependent on external conditions such as ambient temperature (Jirjis 1995). The properties of stored wood material change due to processes such as hydrolysis, autoxidation or microbial degradation (Casal et al. 2010). Deterioration may be dependent on the properties of the material, such as chemical structure, moisture content and availability of nutrients or on the form of storage, such as size of the pile, compacting or covering (Hogland and Marques 2003; Lehtikangas 2000; Slaven et al. 2011). The availability of water to microbes is essential in promoting fermentation and initiating the growth of bacteria (Li et al. 2006). The major losses of chip piles are reportedly due to high temperatures and combustion (Ferrero et al. 2009; Fuller 1985; Li et al. 2006; Tansey 1971). During the first five to seven days of storage the living cells in the wood remain viable and continue to respire resulting in heat generation, with the highest temperature reported at the centre of the pile (Fuller 1985; Nurmi 1999). When temperatures of 60 - 70°C are reached, a chemical reaction occurs in which cellulose molecules are deacetylated forming acetic acid. The rise in acidity and heat causes the wood to darken and ultimately disintegrate as if burned (Fuller 1985). The use of these wood chips in the 'cooking' process result in low yields and high pulpscreen rejects.

Recommendations for chip pile management are; maintaining pile height below 15 m to prevent compaction and allow heat produced during microbial metabolism to be released, preclude tractor spreading of recently delivered chips as this could create fines (sawdust) which contribute to compaction and the trapping of heat, regular monitoring of pile temperature, ensuring wood material is not damp, and species with different deterioration rates should be mixed as needed, to prevent the creation of a large zone with high temperatures due to clumping of chips with high deterioration rates (Fuller 1985; Hogland

and Marques 2003; Li et al. 2006). Modern mills have automated chip pile management equipment facilitating rotation of the pile at regular periods (Quillin 1994). However others rely on a management strategy of first-in-first-out (FIFO). A proper FIFO system involves chip reclaim from the bottom of the chip pile (Quillin 1994), which is most often not the case at commercial pulping mills. A survey conducted in 2000 of 80 United States pulp and paper mills revealed 55% of mills reporting a chip turnover rate in excess of 30 days, 30% reported turnover rates averaging 30 days, whilst 15% recorded weekly pile rotation. In the United States, chip screening is routinely employed after pile storage, a popular practice that is believed to camouflage poor procedures elsewhere in the woodyard. Nevertheless, no system can amend biological or thermal degradation, fungal growth, staining or variations in wood material (Quillin 1994).

Factors such as: the season of wood harvesting, duration of storage, location of stored pile as well as the tree species, particle size and shape of the stored material have been reported as key influences on the quality of stored wood fuels. Inherent properties of the wood such as moisture content, density, calorific value and chemistry may be affected by storage (Brand et al. 2007). These factors may also be pertinent to wood material intended for pulping as conditions for storage are similar. The pulping benefit associated with well-designed and managed storage and reclaim systems is the capacity to deliver standardized chip quality to the mill. A suitably designed wood management system offers regular chip turnover and is inclined to reduce the effects of sporadic chip problems (Quillin 1994). Delivery of high quality chips to the digester will aid in reducing variations in kappa number of the final pulp.

Due to decreasing paper demand and increasing applications for dissolving pulp many paper mills are converting to dissolving pulp production. This type of pulp is comprised of high grade cellulose fibres and can be achieved by limiting cellulose degradation by

eliminating or managing microbial activity. This paper describes the application of an optimized DGGE method to elucidate microflora (bacterial and fungal) indigenous to commercial wood chip piles. A cross sectional survey of the microflora prevalent in summer and winter season is presented together with the chemical properties of wood chips. This is the first such report that correlates the seasonal variation of particular microbes with changes in the wood chemistry.

2. Materials and Methods

2.1 Wood Chip Collection and Processing

Wood chips were obtained from a commercial wood chip pile located in an open woodyard at a pulping mill in Umkomaas, on the south coast of South Africa. Thirty wood chips samples were collected in each of the summer (1-3 February 2011) and winter (2-4 August 2010) seasons from different depths and areas of the wood chip piles to obtain a representative sample (fig. 1). Samples were collected from a section of the woodyard that comprised mainly of *Eucalyptus* spp. (*E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, and *E. smithii*). Three piles were located within this section, the smallest of which was situated between two larger piles (pile 1). In general, the construction of a pile to the height of approximately 20 m requires a week of chipping logs. Samples were collected from five different locations (north, south, east, west, middle) in each area (fig 1: A-L) sampled. Wood chips collected from the different locations for both seasons were discrete samples and may have consisted of different *Eucalyptus* spp. The wood chips in the top layers of each pile were relatively fresh and spent minimal time on the chip pile (1-2 days), the middle layers longer (4-5 days) and the bottom layers of the pile had remained longest (2 weeks). Piles 1 and 3 were located closest to a separating wall which provided some shelter from the elements, whilst pile two was mostly exposed. Samples were collected 0.25 m below the

surface using latex gloves and sterile zip-lock bags (22 x 34 cm) at different depths within the pile. The temperature was recorded at each sampling point. The ambient temperature and humidity were also documented on each sampling day. Samples were transported back to the laboratory and temporarily stored (~5 days) at 4°C until the samples could be milled and stored at -20°C. In accordance with TAPPI T 257 (2012), air-dried wood chips were ground in a Wiley type mill to coarse sawdust. The saw dust was then passed through a 0.40 mm (40 mesh) screen. The milled samples retained in the mesh screen were utilized for DNA extraction (>40 mm). The logistics of sampling from a commercial woodyard presented restrictions as bulldozers were unable to navigate certain piles due to varying pile sizes and heights, therefore collecting samples from exactly the same areas and points of the piles in the different seasons became unachievable. Inevitably samples were collected from different depths and different piles during summer and winter.

2.2 PCR

Genomic DNA was extracted from the milled wood chips using a Soil DNA Extraction kit (Zymo Research), with the following modifications: 0.2 g of sample in 1200 µl of lysis buffer. Ribosomal genes were amplified from microbial genomic DNA from the milled wood chips. Universal primer sets for 16S and the ITS region of the 18S rRNA genes were used and amplification conditions are listed in Table 1. Amplification reactions were composed as previously established by Govender et al. (2013). The volume of DNA was maintained constant in order to establish a standardised method to monitor community changes over time and season. PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, United States). The amplicons were analyzed by electrophoresis on 1% agarose (SeaKem, United States) gels in 1× Tris-Acetate EDTA running buffer at 90 V for 45 min. After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide and visualized

using the Chemi-Genius 2 BioImaging System (Syngene, United States). Upon confirmation of 16S and 18S amplicons, products were purified using a GeneJET™ PCR purification kit (Fermentas, Lithuania) and re-amplified in a touchdown thermal profile program using nested PCR and primers with GC-clamps (Table 1). The composition of the reaction mixtures were the same as that used for the first PCR.

2.3 DGGE

DGGE was performed using the D-Code Universal Mutation Detection System (BioRad, United States), modified from Muyzer et al. (1998). PCR samples were loaded onto vertical perpendicular polyacrylamide gels (6% acrylamidebisacrylamide [37.5:1]) in 1× TAE buffer prepared using 30 and 60% denaturant (100% denaturant corresponds to 7 M urea and 40% formamide). Gradients previously established for optimal separation of bacterial and fungal amplicons with standardized operating conditions were applied (Govender et al. 2013). Bacterial and fungal DNA ladders for DGGE were constructed. They consisted of known microbial species which were isolated, cultured and identified together with bands that were excised, re-amplified and sequenced from other DGGE gels.

2.4 Identification of microorganisms using DGGE

Succeeding PCR and DGGE analysis, DNA was eluted from excised bands and re-amplified using the same primer set excluding the GC-clamp. PCR products were purified and sequenced (Inqaba Biotech, Pretoria). Sequence data were edited and submitted to Genbank for comparison to sequences in the database for identification of genus and species (Altschul et al. 1990).

2.5 Chemical Analyses

Chemical characteristics of the wood chips were evaluated by conducting extractive analyses and these included: (i) Soxhlet extraction method for determination of solvent (water insoluble) extractives, determination of "hot water" (water soluble) extractives (waxes, fats, some resins, photosterols, and non-volatile hydrocarbons, low molecular weight carbohydrates, salts, and other water soluble substances); (ii) Gas Chromatography – Mass Spectrometry (GCMS) for the characterisation of lignin; (iii) Near Infra red reflectance analyser (NIRA) for the rapid quantification of wood chemical components; and (iv) High Performance Liquid Chromatography (HPLC) for quantification of cellulose and hemicelluloses (glucose, mannose, arabinose, xylose, rahamnose, and galactose) (TAPPI Test Methods 1996-1997; Wallis et al. 1996; Wright and Wallis 1996).

2.6 Statistical Analyses

Data was analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. Probability values less than 5% ($p<0.05$) were considered significant.

3. Results

Average ambient summer and winter temperatures and humidity were 25 and 15°C and 64% and 52%, respectively. Temperatures within the various layers of the piles ranged between 22 to 50°C.

3.1 Analysis of Microbial Populations

DGGE profiles of the microbial community (bacteria and fungi) in wood chip samples are shown in figures 2 and 3. A range of amplicons representing bacterial and fungal strains

with varying intensity based on areas and seasons sampled is evident with greater diversity in summer. Seventy nine (45 in summer and 34 in winter) and 29 (20 in summer and 9 in winter) distinct bacterial and fungal amplicons, respectively, were apparent using the optimized DGGE method. The majority of DGGE amplicons were sequenced and identities assigned to 21 and 28 bacteria and 6 and 10 fungi in summer and winter, respectively (table 3 and 4). Variable patterns were observed for different locations of the pile sampled as demonstrated by the variation in the number of bands for each sample, degree of intensity of bands, the emergence or disappearance of bands and their prevalence. A greater diversity of species was observed in samples located in the middle areas. Uncultured *Streptococcus* sp., *Bacillus* sp., *Klebsiella pneumoniae* and *Klebsiella variicola* (fig 2: bands D, E, F, G) appeared together and were consistent throughout the second sampling area in winter, however, these populations varied thereafter with the absence of certain species. Uncultured *Streptococcus* sp. (fig 2: band D) was one of the more prevalent bacterial species however the intensity of its population density varied from area to season. During winter *Bacillus* sp. was one of the predominant species (fig 2: band E).

Pantoea rodasii (fig 2: band A) maintained a consistently high abundance in summer, but was less abundant and varied in the different sampling areas in winter. *Inquilinus limosus* (fig 2: band B) was observed in multiple samples in summer and winter, however, its population was diminished in samples collected from the same area (fig 1: area A and G) in both seasons. *Leuconostoc palmae* was only present in the north, south and east locations (fig 2: band C) of area C in pile one in winter (fig 1), however in summer this population disappeared from pile one (area I) and appeared in pile two (area J) which had greater exposure to the elements (fig 1).

Fewer fungal species were observed compared to the bacterial profiles (Figure 3). A similar pattern was apparent for both seasons and the areas sampled with two predominant

fungal populations, uncultured *Diversispora* sp. (fig 3: band B) and uncultured *Boletaceae* sp. (fig 3: band C) consistent in their appearance and intensity in summer and winter. However, greater diversity amongst the fungal populations was observed in summer. Multiple faint bands identified as uncultured *Scutellospora* sp. (fig 3: band D) and *Ophiostoma bicolour* (fig 3: band F) were observed for samples collected from areas G, H and J of piles 1 and 2 in summer. *Bullera* sp. (fig 3: band A) was identified in the wood chip piles in winter and was represented by intensely bright bands, however, its prevalence was decreased in summer. *Paeciliomyces formosus* was present in several winter and one summer sample. *Phialophora alba* was identified in most of the areas sampled in summer whereas *Phanerochaete chrysosporium* was only identified in one sample in summer.

3.2 Chemical Analyses

The variation in cellulose, hot water extractives, arabinose and galactose levels could be significantly correlated to the different areas in the pile ($p<0.05$) (Figure 4). Significant correlations could also be made between hot water extractives, cellulose, acid insoluble lignin, glucose and season. Cellulose content was higher in winter (44.2%) (Table 5). Hot water extractives were lower in winter (4%) and lowest from the south location of the piles (3.8%). Hot water extractives were highest in the middle (4.2%) and west (6.2%) location of the piles in winter and summer, respectively. Acid insoluble lignin (AIL) levels were significantly greater in winter with the highest percentage from the east location of the piles (26.9%) ($p<0.05$).

By excluding location as a variable and averaging the data for the different depths (A-L), changes in the chemistry of the wood chips were more evident (fig 5). Highest cellulose content was recorded at area F (44.6%) and lowest glucose content at area C (49.3%) in winter. The lowest level of hot water extractives was observed in area D (3.6%) in winter,

indicating lowest lipophilic content in the wood chips at this point. Highest percentage of glucose (50.7%) was evident in area J and lowest cellulose content in area H (43.3%) in summer. Averaged data for both seasons gives a generalized representation of the chemical composition of the wood chips (table 2). Significantly higher glucose (50.18%) and hot water extractives (5.67%) were noted for summer whilst cellulose (44.10%), acid insoluble lignin (26.54%) and xylose (10.86%) appeared to be higher in winter.

4. Discussion

In this cross sectional study an optimized DGGE method (Govender et al. 2013) was used to determine microbial diversity in summer and winter in commercial wood chip piles composed predominantly of *Eucalyptus* species (*E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. smithii*). A greater diversity of bacterial species were present compared to fungi. This is not uncommon as previous reports have documented the succession of microorganisms colonizing wood chips or compost to begin with bacteria followed by rapid colonization by fungi which utilize bacterial metabolic products as growth factors (Clausen 1996; Fuller, 1985). The wood chips in this study had been stored between one to two weeks (1 week in the upper section and 2 weeks in the lower section). In other longitudinal studies and in those with extended storage times (Brand et al. 2007, 2010; Jirjis et al. 2008; Raberg et al. 2009) microbial succession describing a wide variety of bacteria and fungi and greater diversity was described. The greater diversity and abundance of both bacterial and fungal species observed in summer may be accredited to the warmer ambient temperatures (25ºC) and humidity (64%) during this period.

The predominant bacterial population in summer was identified as *P. rodasii*, a recently classified novel gram-negative, facultatively anaerobic plant pathogen previously isolated from *Eucalyptus* seedlings exhibiting symptoms of bacterial blight dieback (Brady et

al. 2012). This implies that the wood species constituting the chip piles in summer were infected with this pathogen which causes dieback of young shoots and leaf blight, ultimately leading to stunting of growth (Coutinho et al. 2002). Previous reports attributed stunted tree growth to a reduction of >20% lignin content (Pilate et al, 2002; Voelker et al. 2010). The lower acid insoluble lignin content recorded in summer correlates significantly to the prevalence of *P. rodasii* compared to winter.

The presence of *I. limosus* is highly unusual as this species is commonly isolated from the respiratory secretions of cystic fibrosis patients (Coenye et al. 2002; Herasimenka et al. 2007). However, *I. ginsengisoli* the only other species in this genus, was isolated from soil from a ginseng field, has a 98.9% similarity to strains of *I. limosus* (Jung et al. 2011). The isolate in this study had a 99% similarity to *I. limosus*.

The natural ecological niche of *Leuconostoc* strains are green vegetables and their roots. *L. palmae* known to be a lactic acid bacterium, is capable of high growth rates and produces lactic acid with high productivities (Yokoyama 2008). Lactic acid bacteria produce a variety of antimicrobial compounds, such as bacteriocins. These compounds are capable of inhibiting many Gram-positive bacteria, which could have an antagonistic effect on the other bacterial populations present (Mataragas et al. 2003).

Common bacterial species such as *Streptococcus* sp., *Bacillus* sp. and *Klebsiella* spp. identified in this study have been reported elsewhere (Clausen 1996; Greaves 1971). Most *Bacillus* species produce cellulases, enzymes that alter the permeability of wood by opening up the crystalline arrangement of the cellulose fibres (Maki et al. 2009)., promoting the diffusion and degradation of other lignocellulolytic enzymes (Clausen 1996). The retention of cellulose is vital for the production of high quality pulp, particularly for dissolving pulp (>90% cellulose) (Christov et al. 1998). The presence of a moderate *Bacillus* sp. population may be beneficial in biopulping processes. However, predominant populations consisting of

more than one *Bacillus* spp. or other cellulolytic genera would impact negatively on cellulose yield. The decreased cellulose content in summer in sample areas A and E compared to B can be significantly associated with increased populations of *Bacillus* spp.

The occurrence of *O. bicolour* may have positive implications as other species of *Ophiostoma* are currently being used to control pitch deposit formation (Held et al. 2003; Su et al. 2011). *Ophiostoma piliferum* a commercialized strain known as Cartapip^{TM}, is successful in reducing pitch in softwood (Blanchette et al. 1992; Farrell et al. 1993; Fischer et al. 1994), however, it is ineffective in removing lipophilic compounds of hardwoods such as *Eucalyptus* species. Reports show that the free sitosterol content tended to increase with treatments of this fungus (Gutiérrez et al. 1999). Su et al. (2011) found the most effective strain in the treatment of Eucalypts to be a variant of *Ophiostoma querci*. However, the lower hot water extractives observed in this study cannot be attributed to the metabolism of *O. bicolour* as lower hot water extractives were noted in winter when this fungus was not detected. Pitch deposits are also known to be lessened (decrease in resin) by storage of the wood as chips in an open wood yard (Gutiérrez et al. 2009). The lowest levels of hot water extractives observed in the south and west locations of the piles in winter may be attributed to greater exposure of these locations to the natural elements.

Lignin content is of concern in the pulping industry since a critical element of pulping is to dissolve and remove lignin fibres from the cell wall to free the cellulose fibres which would facilitate the downstream bleaching process (Oluwadare and Asagbara 2008). Higher acid insoluble lignin levels were observed in winter which may be attributed to the lack of microbial activity and degrading enzymes. Lower cellulose content and higher percentage glucose in summer (table 2) may be correlated with greater microbial activity as the increased glucose level is an indication of the breakdown of cellulose. This reduction in cellulose and increase in glucose may be associated with the greater diversity of indigenous bacterial

species in summer known to produce cellulases which degrade cellulose fibres in wood (Clausen 1996).

This survey of a cross section of wood chips in summer and winter successfully demonstrates that there is seasonal variation in the microflora within the chip pile. Significant variations in wood chemistry based on season and position in pile were observed and some of these variations were significantly correlated to microflora. One of the drawbacks to this study is that in such a mixed wood pile, correlations while significant cannot be absolute as variations contributed by individual wood species cannot be ignored or underestimated. Also while the impact on the quality of pulp may be predicted, actual pulping quality data is lacking. The sampling strategy for this study was not ideal due to the constraints of sampling from a fully operational commercial woodyard where mill operations predominate over scientific research.

Complete data on microflora indigenous to individual *Eucalptus* sp., wood chemistry and pulp quality as well as data after exposure to simulated seasons will allow for greater precision in predicting pulp quality. Future studies that include individual wood species and those incubated in simulation seasonal studies, in conjunction with wood chemistry and final pulp quality and yields may assist in a greater understanding and development of strategies for the potential management of commercial wood chip piles.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215:403-410. doi: 10.1016/S022-2836(05)80360-2

Blanchette RA, Burnes T A, Eerdmans MM, Akhtar M (1992) Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. Holzforschung 46:109-115.

Brady CL, Cleenwerck I, van der Westhuizen L, Venter SN, Coutinho TA, De Vos P (2012) *Pantoea rodasii* sp. nov., *Pantoea rwandensis* sp. nov. and *Pantoea wallisii* sp. nov., isolated from *Eucalyptus*. International Journal of Systematic and Evolutionary Microbiology 62:1457-1464. doi: 10.1099/ijs.0.032615-0

Brand MA, de Muñiz GIB, Quirino WF, Brito JO (2007) Storage as a tool to improve wood fuel quality. Biomass and Bioenergy 35:2581-2588.

Casal MD, Gil MV, Pevida C, Rubiera F, Pis JJ (2010) Influence of storage time on the quality and combustion behaviour of pine woodchips. Energy 35:3066-3071. doi: 10.1016/j.energy.2010.03.048

Christov LP, Akhtar M, Prior BA (1998) The potential of bisulfite pulping in dissolving pulp production. Enzyme and Microbial Technology 23:70. doi: 10.1016/S0141-0229(98)00017-9

Clausen CA (1996) Bacterial associations with decaying wood: a review. International Biodeterioration & Biodegradation 37:101-107. doi: 10.1016/0964-8305(95)00109-3

Coenye T, Goris J, Spilker T, Vandamme P, LiPuma JJ (2002) Characterization of unusual bacteria isolated from respiratory secretions of cystic fibrosis patients and description of *Inquilinus limosus* gen. nov., sp. nov. Journal of Clinical Microbiology 40:2062-2069. doi: 10.1128/JCM.40.6.2062-2069.2002

Coutinho TA, Preisig O, Mergaert J, Cnockaert MC, Riedel K, Swings J, Wingfield MJ (2002) Bacterial blight and dieback of *Eucalyptus* species, hybrids, and clones in South Africa. Plant Disease 86:20-25. doi: 10.2094/PDIS.2002.86.1.20

Farrell RL, Blanchette RA, Brush TS, Hadar Y, Iverson S, Krisa K, Wendler PA, Zimmerman W (1993) Cartapip^{TM}: a biopulping product for control of pitch and resin acid problems in pulp mills. Journal of Biotechnology 30:115-122. doi: 10.1016/0168-1656(93)90032-I

Fischer K, Akhtar M, Blanchette RA, Burnes TA, Messner K, Kirk TK (1994) Reduction of resin content in wood chips during experimental biological pulping processes. Holzforschung 48:285-290. doi: 10.1515/hfsg.1994.48.4.285

Fuller WS (1985) Chip pile storage – a review of practices to avoid deterioration and economic losses. Tappi Journal 68:48-52.

Ferrero F, Lohrer C, Schmidt BM, Noll M, Malow M (2009) A mathematical model to predict the heating-up of large scale wood piles. Journal of Loss Prevention in the Process Industries 22:439-448. doi: 10.1016/j.jlp.2009.02.009

Govender L, Bush T, Govinden R (2013) Method optimization for DGGE analysis of microflora from industrial wood chips intended for pulping. Online International Journal of Microbiology Research, *In press*

Greaves H (1971) The bacterial factor in wood decay. Wood Science and Technology 5:6-16. doi: 10.1007/BF00363116

Gutiérrez A, del Río JC, Martínez AT (2009) Microbial and enzymatic control of pitch in the pulp and paper industry. Applied Microbiological Biotechnology 82:1005-1018. doi: 10.1007/s00253-009-1905-z

Gutiérrez A, del Rio JC, Martinez MJ, Martinez AT (1999) Fungal degradation of lipophilic extractives in *Eucalyptus globulus* wood. Applied Environmental Microbiology 4:1367-1371. doi: 0099-2240/99/$04.00+0

Handschur M, Piñar G, Gallist B, Lubitz W, Haslberger AG (2005) Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. Food and Chemical Toxicology 43:1595-1605. doi: 10.1016/j.fct.2005.05.009

Held BW, Thwaites JM, Farrell RL, Blanchette RA (2003) Albino strains of *Ophiostoma* species for biological control of sapstaining fungi. Holzforschung 57:237-242. doi: 10.1515/HF.2003.036

Herasimenka Y, Cescutti P, Impallomeni G, Rizzo R (2007) Exopolysaccharides produced by *Inquilinus limosus*, a new pathogen of cystic fibrosis: novel structures with usual components. Carbohydrate Research 342:2404-2415. doi: 10.1016/j.carres.2007.07.012

Hogland W, Marques M (2003) Physical, biological and chemical processes during storage and spontaneous combustion of waste fuel. Resources, Conservation and Recycling 40:53-69. doi:10.1016/S0921-3449(03)00025-9

Ji N, Peng B, Wang G, Wang S, Peng X (2004) Universal primer PCR with DGGE for rapid detection of bacterial pathogens. Journal of Microbiological Methods 57:409-413. doi: 10.1016/j.mimet.2004.02.010

Jirjis R, Pari L, Sissot F (2008) Storage of poplar wood chips in Northern Italy. World Bioenergy 8:85-89.

Jirjis R (1995) Storage and drying of wood fuel. Biomass and Bioenergy 9:181-190.

Jung H.-M, Lee J-S, Bae H-M, Yi T-H, Kim S-E, Lee S-T, Im W-T (2011) *Inquilinus ginsengisoli* sp. nov., isolated from soil of a ginseng field. International Journal of Systematic and Evolutionary Microbiology 61:201-204. doi: 10.1099/ijs.0.018689-0

Lehtikangas P **(**2000) Storage effects on pelletised sawdust, logging residues and bark. Biomass and Bioenergy 19:287-293. doi:10.1016/S0961-9534(00)00046-5

Li X, Koseki H, Momota M (2006) Evaluation of danger from fermentation-induced spontaneous ignition of wood chips. Journal of Hazardous Materials A135:15-20. doi: 10.1016/j.jhazmat.2005.11.034

Maki M, Leung KT, Qin W (2009) The prospects of cellulose-producing bacteria for the bioconversion of lignocellulosic biomass. International Journal of Biological Sciences 5:500-516. doi: 10.7150/ijbs.5.500

Marchesi JR, Sato T, Weightman AJ, Fry JC, Hiom SJ (1998) Design and evaluation of useful bacterial-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology 64:795-799.

Mataragas M, Metaxopoulos, Galiotou M, Drosinos EH (2003) Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. Meat Science 64:265-271. doi: 10.1016/S0309-1740(02)00188-2

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73:127-141. doi: 10.1023/A:1000669317571

Nurmi J (1999) The storage of logging residue for fuel. Biomass and Bioenergy 17:41-47. doi: 10.1016/S0961-9534(99)00023-9

Oluwadare AO, Asagbara EO (2008) Biodegradation of *Sterculia setigera* (Sterculiaceae) chips and its effects on wood basic chemical composition. International Journal of Botany 4:461-465.

Pilate G, Guiney E, Holt K, Petit-Conil M, Lapierre C, Leplé J, Pollet B, Mila I, Webster EA, Marstorp HG, Hopkins DW, Jouanin L, Boerjan W, Schuch W, Cornu D, Halpin C (2002) Field and pulping performances of transgenic trees with altered lignification. Natural Biotechnology 20:607-612. doi: 10.1038/nbt0602-607

Pogue TE (2008) Wood, paper and pulp. Final report commissioned by the Department of Labour for the Sector skills research project. Pretoria: Human Sciences Research Council pp 132-149.

Quillin S (1994) Effective chip pile storage design reduces pulp variation, improves mill profits. Pulp and Paper magazine issue: 940201. Available at: http://www.risiinfo.com/db_area/archive/p_p_mag/1994/9402/94020111.htm

Sappi-Chemical Cellulose (2007) The Paper Making Process: From wood to coated paper. Sappi Europe SA, Accessed: June 2010. Available at http://www.google.co.za/url?sa=t&rct=j&q=the%20paper%20making%20process%3A%20from%20wood%20to%20coated%20paper.%20sappi%20europe%20sa&source=web&cd=1&cad=rja&ved=0CC0QFjAA&url=http%3A%2F%2Fwww.na.sappi.com%2Fdocuments%2F10165%2F13508%2FThe%2BPapermaking%2BProcess.pdf&ei=YldJUZ7uDtO5hAep_oHwBg&usg=AFQjCNEAVE4i5vYUqXZZw8CtGBYFITjf3g

Schabereiter-Gurtner C, Piñar G, Lubitz W, Rölleke S (2001) Analysis of fungal communities on historical church window glass by denaturing gradient gel electrophoresis and phylogenetic 18S rDNA sequences analysis. Journal of Microbiological Methods 47:345-354.

Slaven I, Haviarova E, Cassens D (2011) Properties of wood waste stored for energy production. BioEnergy ID-421-W. Available at: http://works.bepress.com/isaac_slaven/4

Su Y, Ho C, Hsu K, Chang H, Farrell R, Wang EI (2011) Screening of *Ophiostoma* species for removal of *Eucalyptus* extractives. Journal of Wood Chemistry and Technology 31:282-297. doi: 10.1080/02773813.2010.523164

Tansey MR (1971) Isolation of thermophilic fungi from self-heated, industrial wood chip piles. Mycologia 63:537-547.

TAPPI (2012) Test Methods T 257 cm-12. Sampling and preparing for wood analysis. Tappi Test Methods, Tappi Press, Atlanta.

TAPPI (1996) Test Methods T-204-OM-88: Solvent extractives T-222-OM-88: Acid insoluble lignin of wood and pulp T249 cm-85: Carbohydrate anaylsis. Tappi Test Methods 1996-1997, Tappi Press, Atlanta.

Vainio EJ, Hantula J (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycology Research 104:927-936. doi: 10.1017/S0953756200002471

Voelker SL, Lachenbruch B, Meinzer FC, Jourdes M, Ki C, Patten AM, Davin LB, Lewis NG, Tuskan GA, Gunter L, Decker SR, Selig MJ, Sykes R, Himmel ME, Kitin P, Shevchenko O, Strauss SH (2010) Antisense down-regulation of 4CL expression alters lignification, tree growth, and saccharification potential of field-grown poplar. Plant Physiology 154:874-886.

Wallis AFA, Wearne RH, Wright PJ (1996) Chemical analysis of polysaccharides in plantation eucalypt woods and pulps. Appita Journal 49(4):258-262.

White TJ, Burns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ, (ed) PCR Protocols, Academic Press, San Diego, CA, pp 315-322.

Wright PJ, Wallis AFA (1996) Rapid determination of carbohydrates in hardwoods. Holzforschung 50:518-524. doi: 10.1515/hfsg.1996.50.6.518

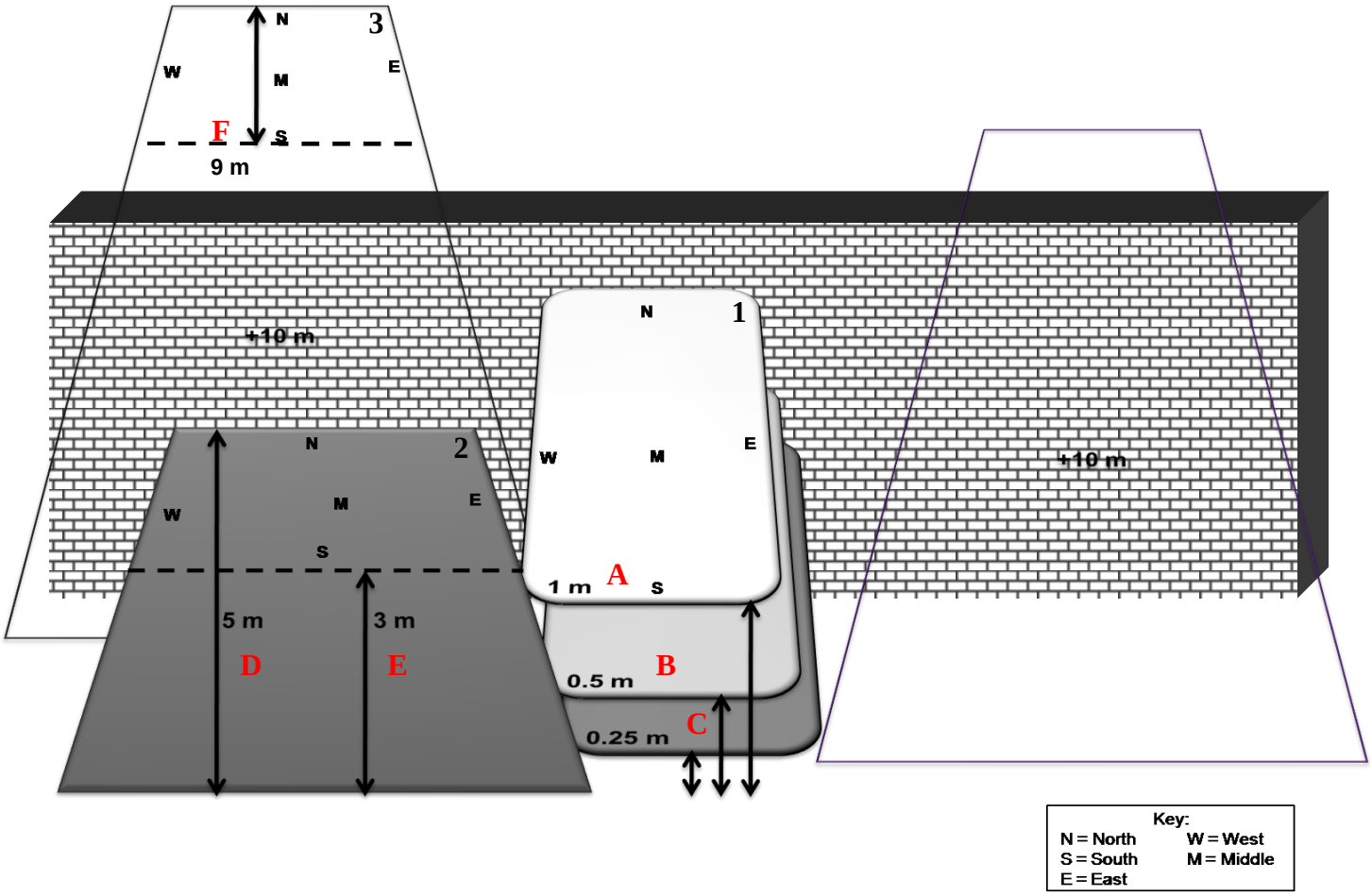
Yokoyama S (2008) The Asian biomass handbook. The Japan Institute of Energy, Japan.

Table 1 Primers and PCR reaction conditions for amplification of 16S and the ITS region of 18S ribosomal genes

Primer name	Nucleotide sequence (5'-3')	Amplicon length (bp)	Techniques	Reaction Conditions	Reference
63F	CAGGCCTAACACATGCAAGTC	1300	PCR	Initial denaturation: 95°C, 5' 30 Cycles: Denaturation: 95°C, 1' Annealing: 55°C, 1' Extension: 72°C, 1.5' Final Extension: 72°C, 5'	(Marchesi et al. 1998)
1387R	GGCGGWGTGTACAAGGC				
ITS5F	GGAAGTAAAAGTCGTAACAAGG	600	PCR	Initial denaturation: 95°C, 2' 25 Cycles: Denaturation: 95°C, 30'' Annealing: 53°C, 45'' Extension: 72°C, 1' Final Extension: 72°C, 8'	(Vainio and Hantula 2000; White et al. 1990)
ITS4R	CCTCCGCTTATTGATATGCTTAAG				
338F*	ACTCCTACGGGAGGCAGCAG	237	PCR-DGGE	Initial denaturation: 94°C, 5' 30 Cycles: Denaturation: 95°C, 1' Annealing: 53°C, 1' Extension: 72°C, 2' Final Extension: 72°C, 5'	(Handschur et al. 2005)
518R	ATTACCGCGGCTGCTGG				
933F*	GCACAAGCGGTGGAGCATGTGG	500	PCR-DGGE		(Ji et al. 2004)
1387R	GCCCGGGAACGTATTCACCG				
NS26F	CTGCCCTATCAACTTTCGA	316	PCR-DGGE	Initial denaturation: 94°C, 5' 30 Cycles: Denaturation: 95°C, 1' Annealing: 55°C, 1' Extension: 72°C, 1' Final Extension: 72°C, 5'	(Schabereiter-Gurtner et al. 2001)
518R*	ATTACCGCGGCTGCTGG				
EF4F	GGAAGGGGTGTATTTATTAG	426	PCR-DGGE		(Schabereiter-Gurtner et al. 2001)
518R*	ATTACCGCGGCTGCTGG				

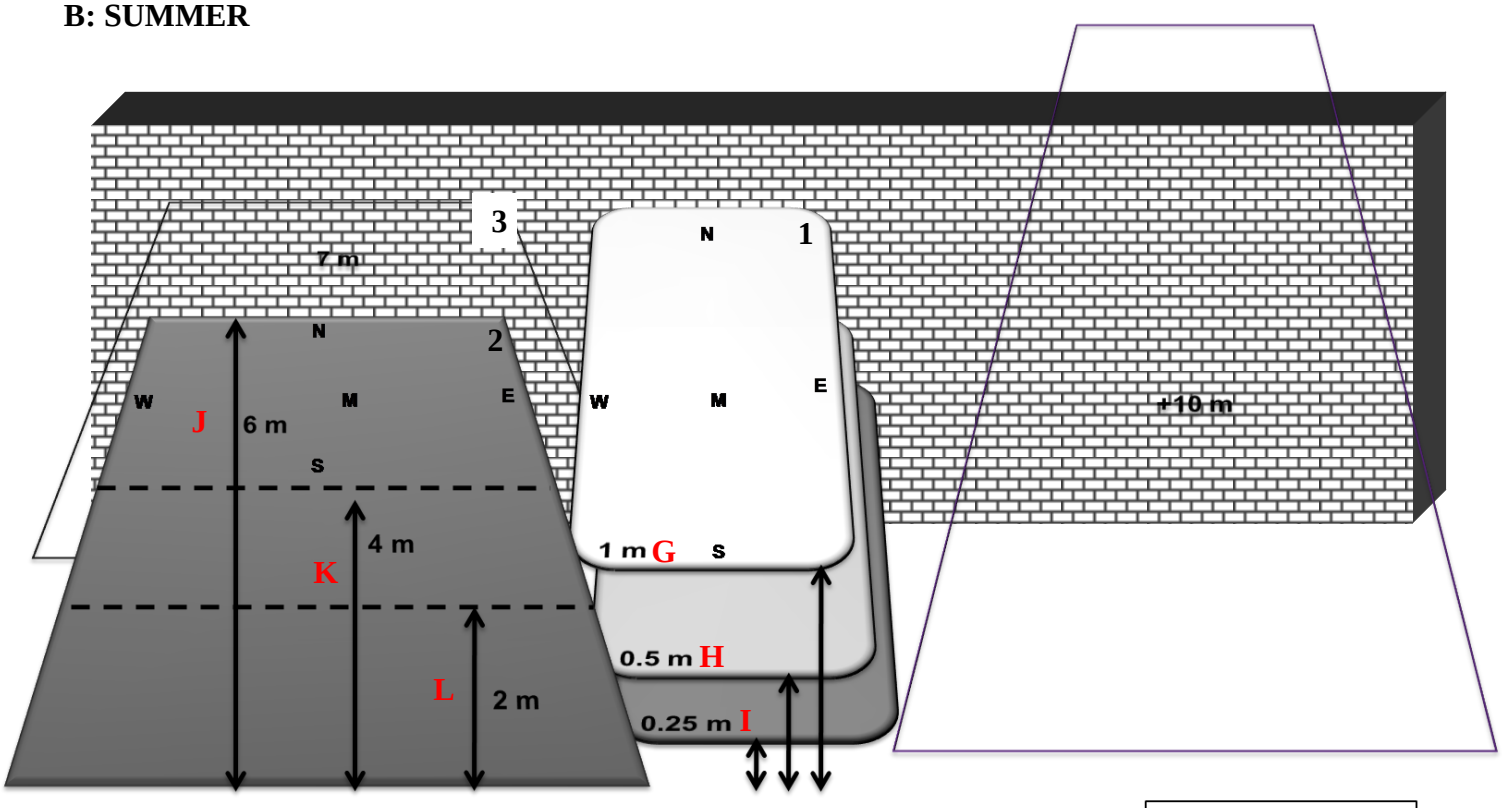
*GC-clamp added to the 5´ end of the primer (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´)

A: WINTER



Key:
N = North W = West
S = South M = Middle
E = East

B: SUMMER



Key:
N = North W = West
S = South M = Middle
E = East

Fig. 1 Schematic layout of the wood chip piles at a commercial pulping mill and sampling points in summer and winter. Piles: 1-3, areas: A-L, locations: north, south, east, west, middle

Table 2 Sampling points, dates and temperature at specified depth

	Date	Sample No.	Location	Area	Depth (m)	Temperature (°C)	Ave Temp (°C)
WINTER	02 August 2010 Ambient temperature: **15ºC** Humidity: **55%**	1	North	A	0.25	30	31.4
		2	South			28	
		3	East			27	
		4	West			22	
		5	Middle			50	
	03 August 2010 Ambient temperature: **15.2ºC** Humidity: **52%**	6	North	B	0.5	35	39
		7	East			45	
		8	South			40	
		9	Middle			42	
		10	West			33	
		11	Middle	C	0.75	27	34
		12	North			48	
		13	West			34	
		14	South			34	
		15	East			27	
		16	Middle	D	0.25	25	28.6
		17	North			34	
		18	West			28	
		19	South			28	
		20	East			28	
	04 August 2010 Ambient temperature: **15.4ºC** Humidity: **50%**	21	South	E	2.25	41	32.5
		22	Middle			32	
		23	North			29	
		24	West			30	
		25	East			30	
		26	North	F	1.25	30	29.6
		27	South			31	
		28	Middle			31	
		29	East			28	
		30	West			28	
SUMMER	01 February 2011 Ambient temperature: **25ºC** Humidity: **66%**	31	North	G	0.275	44	36
		32	South			33	
		33	East			39	
		34	West			33	
		35	Middle			31	
		36	North	H	0.5	32	33.6
		37	South			39	
		38	East			31	
		39	West			35	
		40	Middle			31	
	02 February 2011 Ambient temperature: **25.2ºC** Humidity: **65%**	41	North	I	0.75	34	31.6
		42	South			31	
		43	East			31	
		44	West			32	
		45	Middle			30	
		46	North	J	0.25	34	37.4
		47	South			41	
		48	East			33	
		49	West			33	
		50	Middle			46	
	03 February 2011 Ambient temperature: **25.3ºC** Humidity: **62%**	51	North	K	2.25	34	33
		52	South			35	
		53	East			31	
		54	West			31	
		55	Middle			34	
		56	North	L	4.25	34	36.8
		57	South			36	
		58	East			34	
		59	West			48	
		60	Middle			32	

Table 3 Bacteria identified by sequencing of 16S rRNA amplicons of excised bands from PCR-DGGE gels

	Sites	Bacterial Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Accession Number
WINTER	A	*Pantoea rodasii*	Proteobacteria	JX113243.1	97	*
		Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Klebsiella pneumonia	Proteobacteria	JF919928.1	97	KC562166
	B	*Klebsiella variicola*	Proteobacteria	JX489160.1	97	KC562165
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Klebsiella pneumonia	Proteobacteria	JX406150.1	98	KC562166
		Sphingomonadaceae sp.	Proteobacteria	AY673323.1	100	#
		Uncultured Lachnospiraceae bacterium	Firmicutes	EF706735.1	100	#
	C	*Klebsiella variicola*	Proteobacteria	JX489160.1	97	KC562165
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Klebsiella pneumonia	Proteobacteria	JX406150.1	98	KC562166
		Shewanella sp.	Proteobacteria	JN391164.1	97	#
		Pantoea agglomerans	Proteobacteria	JF938967.1	97	#
		Uncultured bacterium	No rank	JN214344.1	92	#
		Klebsiella oxytoca	Proteobacteria	CP003683.1	93	#
		Uncultured *Lysobacter sp.*	Proteobacteria	FM175860.1	94	#
		Pantoea rodasii	Proteobacteria	JX113243.1	97	*
		Inquilinus limosus	Proteobacteria	JN592468.1	99	KC562163
	D	*Pantoea rodasii*	Proteobacteria	JX113243.1	97	*
		Inquilinus limosus	Proteobacteria	JN592468.1	99	KC562163
		Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Pseudomonas sp.	Proteobacteria	JN680199.1	99	KC493047
		Bacillus cereus	Firmicutes	JQ389625.1	100	KC493049
		Uncultured bacterium	No rank	JF226237.1	91	#
	E	*Leuconostoc palmae*	Firmicutes	AM940225.1	90	KC562164
		Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Pseudomonas sp.	Proteobacteria	JN680199.1	99	KC493047
		Bacillus cereus	Firmicutes	JQ389625.1	100	KC493049
		Spingomonadaceae sp.	Proteobacteria	AY673323.1	100	#
		Uncultured *Erwinia* sp.	Proteobacteria	KC020167.1	100	KC562161
		Klebsiella oxytoca	Proteobacteria	CP003683.1	93	#
		Uncultured gamma proteobacterium	Proteobacteria	EU810909.1	90	#
		Pantoea rodasii	Proteobacteria	JX113243.1	97	*
		Inquilinus limosus	Proteobacteria	JN592468.1	99	KC562163
	F	*Pantoea rodasii*	Proteobacteria	JX113243.1	97	*
		Inquilinus limosus	Proteobacteria	JN592468.1	99	KC562163
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*
		Pseudomonas sp.	Proteobacteria	JN680199.1	99	KC493047
		Bacillus cereus	Firmicutes	JQ389625.1	100	KC493049
		Methylobacterium sp.	Proteobacteria	GQ342543.1	100	#
		Bacillus cereus	Firmicutes	AM397642.1	100	KC493049
		Leuconostoc palmae	Firmicutes	AM940225.1	90	KC562164
		Klebsiella oxytoca	Proteobacteria	CP003683.1	93	#
SUMMER	G	*Pantoea rodasii*	Proteobacteria	JX113243.1	97	*
		Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*
		Klebsiella oxytoca	Proteobacteria	CP003683.1	93	#
		Sphingomonadaceae sp.	Proteobacteria	AY673323.1	100	#
	H	*Leuconostoc palmae*	Firmicutes	AM940225.1	90	KC562164
		Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*

		Pseudomonas sp.	Proteobacteria	JN680199.1	99	KC493047
		Bacillus cereus	Firmicutes	JQ389625.1	100	KC493049
		Bacillus sp.	Firmicutes	GU325802.1	100	*
	I	*Pantoea rodasii*	Proteobacteria	JX113243.1	97	*
		Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Klebsiella pneumonia	Proteobacteria	JX406150.1	98	KC562166
		Klebsiella variicola	Proteobacteria	JX489160.1	97	KC562165
		Bacillus cereus	Firmicutes	JQ389625.1	100	KC493049
		Erwinia sp.	Proteobacteria	FM161470.1	97	#
		Uncultured bacterium	No rank	JN214344.1	92	#
		Pectobacterium cypripedii	Proteobacteria	EF159725.1	99	#
		Klebsiella oxytoca	Proteobacteria	CP003683.1	93	#
		Methylobacterium sp.	Proteobacteria	GQ342543.1	100	#
		Inquilinus limosus	Proteobacteria	JN592468.1	99	KC562163
	J	*Klebsiella pneumonia*	Proteobacteria	JX406150.1	98	KC562166
		Pantoea rodasii	Proteobacteria	JX113243.1	97	*
		Inquilinus limosus	Proteobacteria	JN592468.1	99	KC562163
		Pantoea eucalypti	Proteobacteria	EF688009.1	96	*
		Erwinia sp.	Proteobacteria	FM161470.1	97	#
		Uncultured bacterium	No rank	JN214344.1	92	#
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Methylobacterium sp.	Proteobacteria	GQ342543.1	100	#
		Pantoea agglomerans	Proteobacteria	JQ513923.1	82	#
		Klebsiella oxytoca	Proteobacteria	CP003683.1	93	#
	K	*Pantoea rodasii*	Proteobacteria	JX113243.1	97	*
		Klebsiella variicola	Proteobacteria	JX489160.1	97	KC562165
		Klebsiella pneumoniae	Proteobacteria	JX406150.1	98	KC562166
		Inquilinus limosus	Proteobacteria	JN592468.1	99	KC562163
		Klebsiella oxytoca	Proteobacteria	CP003683.1	93	#
		Erwinia sp.	Proteobacteria	FM161470.1	97	#
		Methylobacterium sp.	Proteobacteria	GQ342543.1	100	#
	L	Uncultured *Streptococcus* sp.	Firmicutes	HM053796.1	100	*
		Bacillus sp.	Firmicutes	GU325802.1	100	*
		Klebsiella pneumoniae	Proteobacteria	JX406150.1	98	KC562166
		Pseudomonas sp.	Proteobacteria	JN680199.1	99	KC493047
		Bacillus cereus	Firmicutes	JQ389625.1	100	KC493049
		Uncultured bacterium	No rank	JN214344.1	92	#
		Methylobacterium sp.	Proteobacteria	GQ342543.1	100	#
		Pantoea eucalypti	Proteobacteria	EF688009.1	96	#
		Pseudomonas sp.	Proteobacteria	JN680199.1	99	KC493047
		Pantoea rodasii	Proteobacteria	JX113243.1	97	*
		Streptomyces sp.	Actinobacteria	GU132502.1	99	KC493050
		Modestobacter sp.	Actinobacteria	JX982719.1	89	KC493063

*Accession numbers could not be assigned to excised bands that were less than 150 bp after sequencing.
#GenBank accession numbers pending

Table 4 Fungi identified by sequencing of ITS 18S rRNA amplicons of excised bands from PCR-DGGE gels

	Sites	Fungal Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Accession Number
WINTER	A	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
	B	*Bullera* sp.	Basidiomycota	AY313030.1	83	KC562155
		Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Paecilomyces formosus	Ascomycota	GU968673.1	99	JX945649
		Uncultured *Scutellospora* sp.	Glomeromycota	HE613501.1	96	KC562158
		Uncultured Basidiomycota sp.	Basidiomycota	JQ627512.1	88	KC562159
	C	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Paecilomyces formosus	Ascomycota	GU968673.1	99	JX945649
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Uncultured Basidiomycota sp.	Basidiomycota	JQ627512.1	88	KC562159
	D	*Bullera* sp.	Basidiomycota	AY313030.1	83	KC562155
		Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
	E	*Bullera* sp.	Basidiomycota	AY313030.1	83	KC562155
		Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Uncultured *Scutellospora* sp.	Glomeromycota	HE613501.1	96	KC562158
	F	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Uncultured *Scutellospora* sp.	Glomeromycota	HE613501.1	96	KC562158
SUMMER	G	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Paecilomyces formosus	Ascomycota	GU968673.1	99	JX945649
		Uncultured *Scutellospora* sp.	Glomeromycota	HE613501.1	96	KC562158
		Uncultured Basidiomycota sp.	Basidiomycota	JQ627512.1	88	KC562159
		Uncultured Ascomycota	Ascomycota	EU484181.1	99	KC020157.1
	H	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Uncultured *Scutellospora* sp.	Glomeromycota	HE613501.1	96	KC562158
		Uncultured Basidiomycota sp.	Basidiomycota	JQ627512.1	88	KC562159
		Ophiostoma bicolour	Ascomycota	AB007666.1	90	KC562160
		Uncultured Ascomycota	Ascomycota	EU484181.1	99	KC020157.1
	I	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Uncultured *Scutellospora* sp.	Glomeromycota	HE613501.1	96	KC562158
		Phialophora alba	Ascomycota	HM116755.1	99	JX945655
	J	*Bullera* sp.	Basidiomycota	AY313030.1	83	KC562155
		Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Uncultured *Scutellospora* sp.	Glomeromycota	HE613501.1	96	KC562158
		Phialophora alba	Ascomycota	HM116755.1	99	JX945655
		Ophiostoma bicolour	Ascomycota	AB007666.1	90	KC562160
		Uncultured Ascomycota	Ascomycota	EU484181.1	99	KC020157.1
	K	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Phialophora alba	Ascomycota	HM116755.1	99	JX945655
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Phanerochaete chrysosporium	Basidiomycota	AF475147.1	100	JX945648
		Penicillium decumbens	Ascomycota	JQ664732.1	99	KC562162
	L	Uncultured *Diversispora* sp.	Glomeromycota	HE576934.1	96	KC562156
		Uncultured *Boletaceae* sp.	Basidiomycota	EF024378.1	96	KC562157
		Phialophora alba	Ascomycota	HM116755.1	99	JX945655

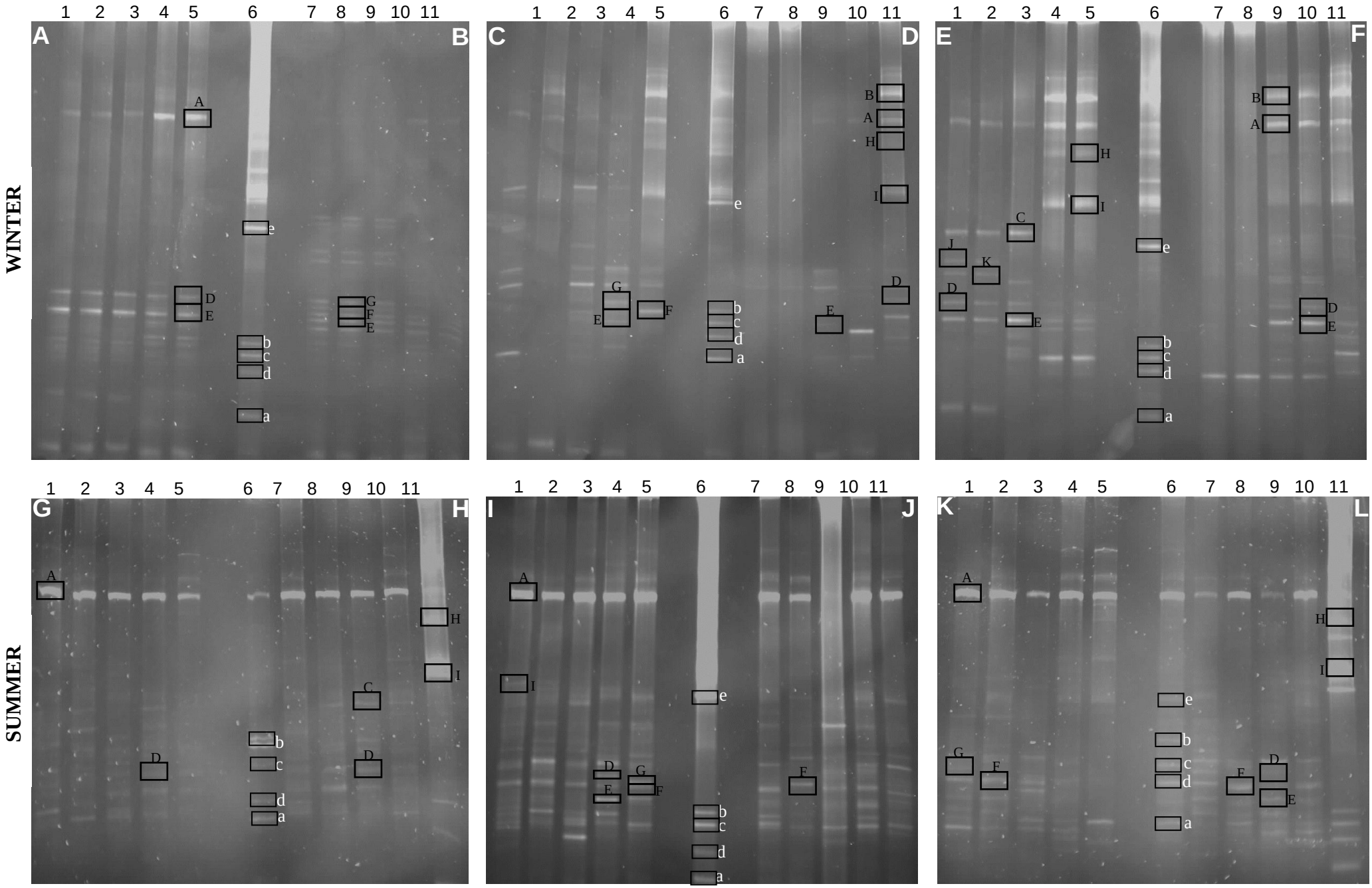


Fig. 2 Bacterial DGGE profiles of wood chip samples collected from the pulping mill in winter (area A-F) and summer (area G-L) (16S). Lanes 1-5: north, south, east, west, middle, lane 6: known marker ([a]*Inquilinus limosus*, [b]*Klebsiella* sp, [c]*Pantoea* sp., [d]*Micrococcus luteus* and [e]*Pseudomonas aeruginosa*), lane 7-11: north, south, east, west, middle. Bands highlighted: D- Uncultured *Streptococcus* sp., E- *Bacillus* sp., F- *Klebsiella pneumonia*, G- *Klebsiella variicola*.

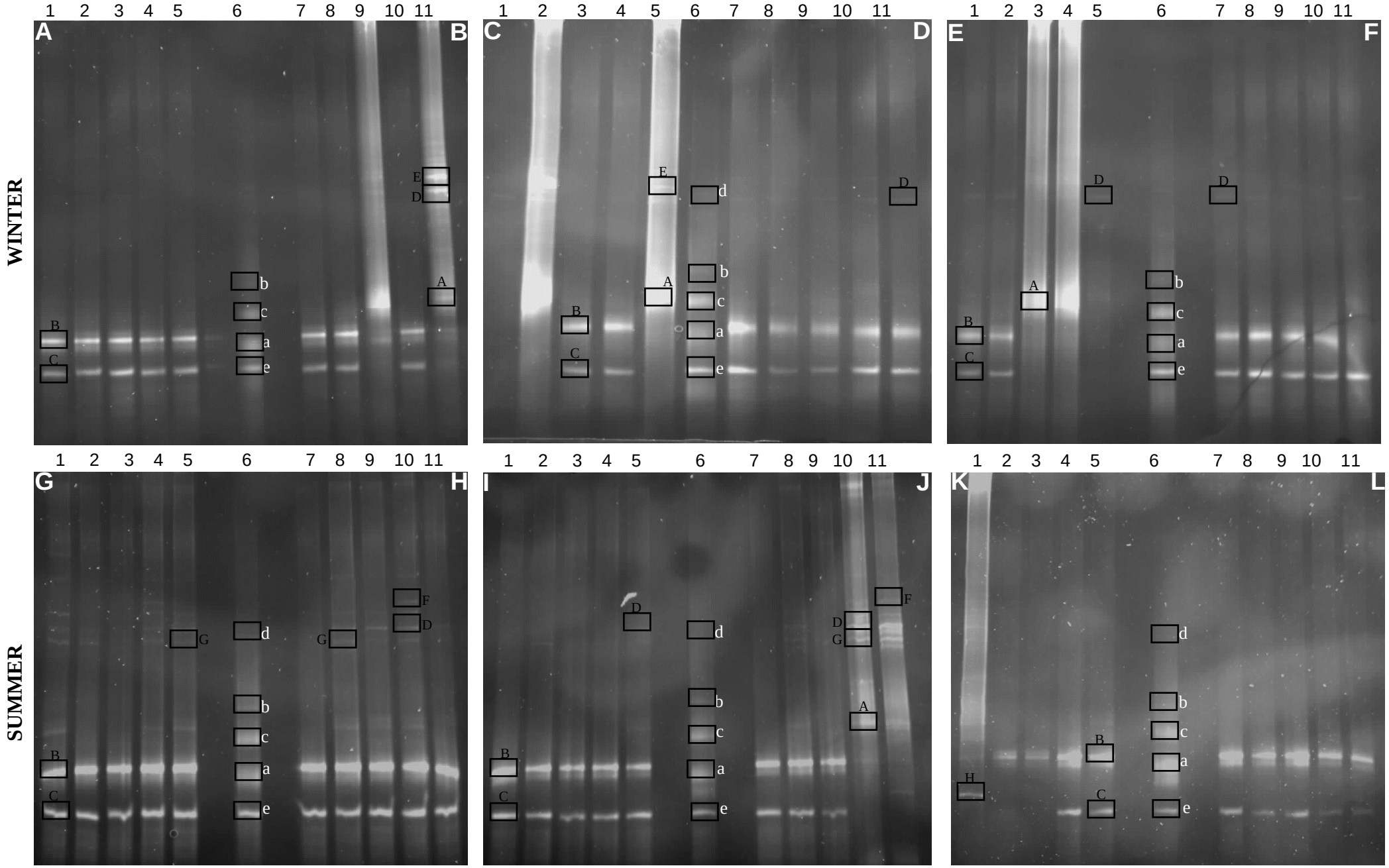
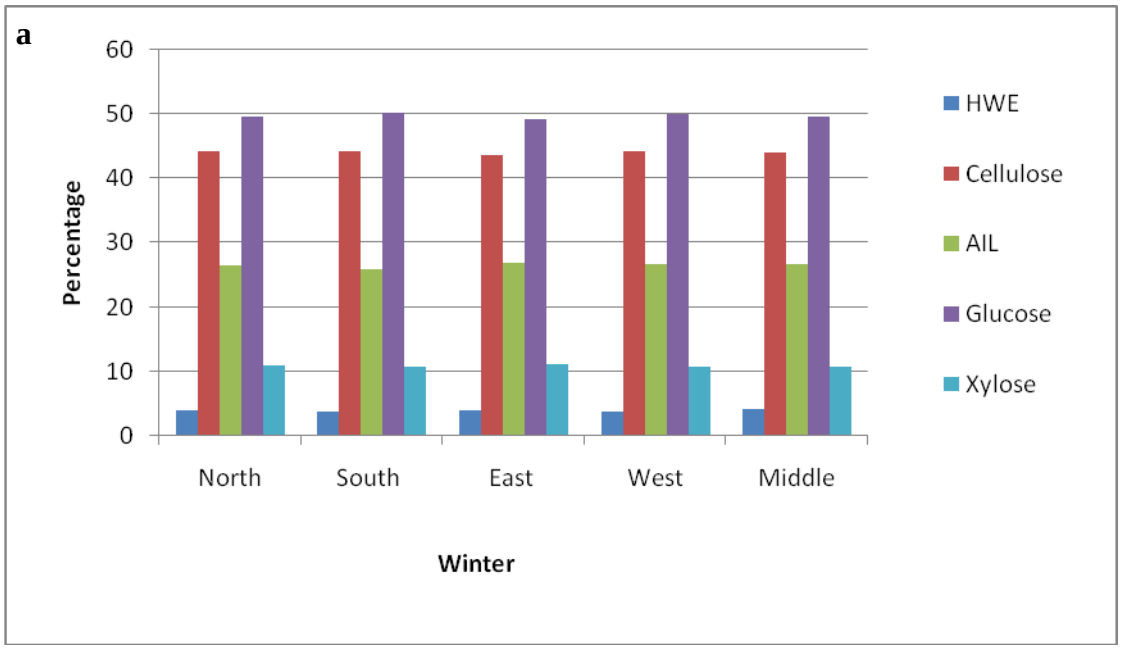


Fig. 3 Fungal DGGE profiles of wood chip samples collected from the pulping mill in winter (area A-F) and summer (area G-L) (18S). Lanes 1-5: north, south, east, west, middle, lane 6: known marker ([a]*Aspergillus fumigatus*, [b]*Paecilomyces formosus*, [c]*Phialophora alba*, [d]*Curvularia* sp. and [e]*Aspergillus fumigatus*), lane 7-11: north, south, east, west, middle. Bands highlighted: A- *Bullera* sp., B- *Diversispora* sp, C- *Boletaceae* sp., D- *Scutellospora* sp.,F- *Ophiostoma bicolour*.



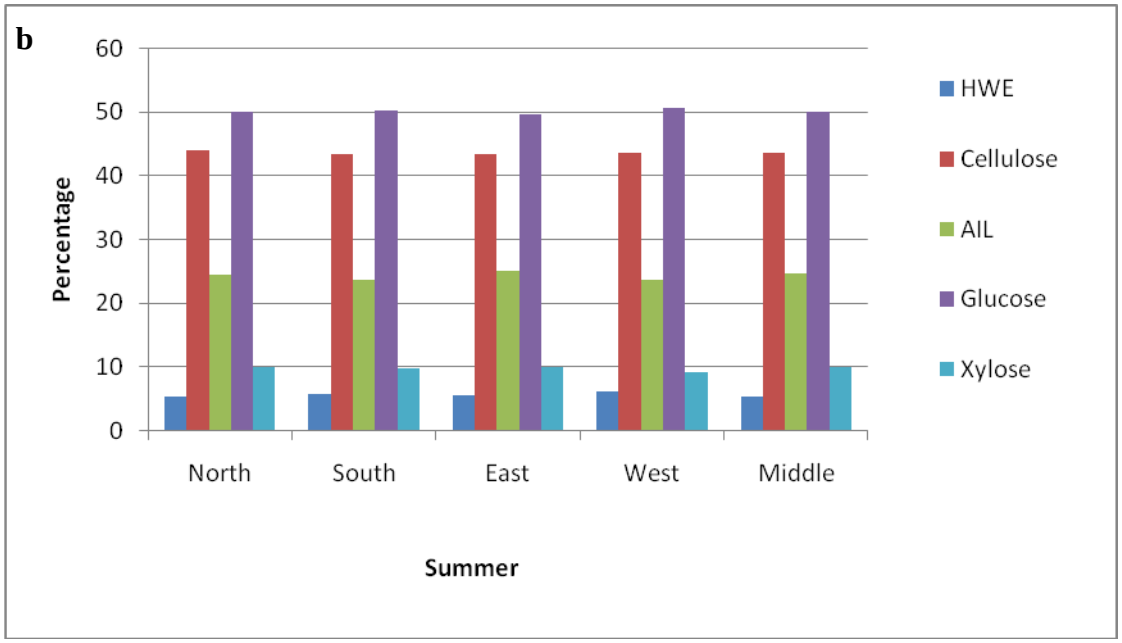


Fig. 4 Chemical analysis of wood chips collected from the pulping mill in winter (a) and summer (b) from the different sampling points. Data averaged for the different locations within each sampling area. HWE = Hot Water Extractives, AIL = Acid Insoluble Lignin.

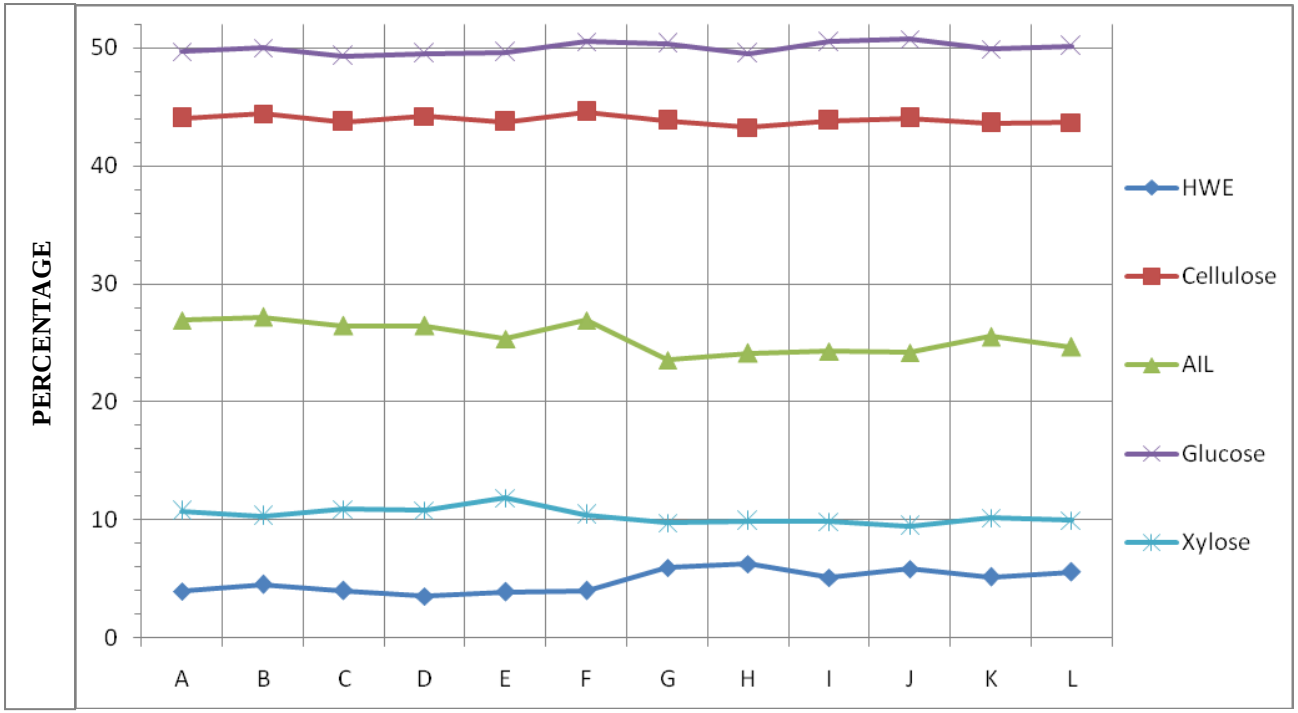


Fig. 5 Chemical analysis of wood chips collected from different areas of the wood chip piles (A-L) at the pulping mill in winter and summer. Each point represents average data of the three replicates each within the five locations sampled. HWE = Hot Water Extractives, AIL = Acid Insoluble Lignin.

Table 5 Averaged chemistry data (of the areas sampled, locations and replicates) of the wood chips collected from the different piles in summer and winter. HWE = Hot Water Extractives, AIL = Acid Insoluble Lignin.

		HWE (%)	**AIL (%)**	**Glucose (%)**	**Xylose (%)**	**Cellulose (%)**
WINTER	Pile 1	4.16	26.86	49.63	10.68	44.04
	Pile 2	3.75	25.88	49.55	11.34	43.96
	Pile 3	4.03	26.91	50.53	10.44	44.56
	Average	**3.98**	**26.55**	**49.90**	**10.82**	**44.19**
SUMMER	Pile 1	5.81	23.99	50.11	9.85	43.63
	Pile 2	5.54	24.79	50.25	9.89	43.77
	Average	**5.67**	**24.39**	**50.18**	**9.87**	**43.70**

Strategic Combination of *Eucalyptus* species for the Potential Management of Pulp Quality

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Keywords wood chips; *Eucalyptus*, pulp yield, lignocellulolytic enzymes, DGGE

Abstract

In South Africa dissolving pulp is manufactured from *Eucalyptus*, pine and wattle. The stored wood chips are vulnerable to degradation due to microbiological metabolism and spontaneous combustion. Use of these chips could result in poor pulp quality. This study describes the microflora indigenous to two *Eucalyptus* species and a combination of the two and determines whether there are any correlations between wood chip quality and chemical and physical properties of the wood species and their microflora before and after exposure to simulated weather conditions. Microbial strains were identified after sequencing of 16S and 18S rRNA amplicons separated by Denaturing Gradient Gel Electrophoresis (DGGE). Fungal and bacterial species were isolated, cultured, identified and screened for lignocellulolytic enzyme activity. Eighty six percent of the fungi isolated were capable of producing cellulase and xylanase. Significant correlations between the microflora, seasons (greater diversity and loading in summer) and the chemical and physical properties of wood chips and pulp (lower cellulose and viscosity in summer) as well as *Eucalyptus* species (significantly higher cellulose and viscosity for the combination and *E. nitens*) were found. Indigenous microflora of each wood species may be one of the contributing factors to poor/good pulp quality. Microbial enzymes and pulp quality and yields were significantly correlated. This investigation provides proof of concept that combining wood with different deterioration rates results in an overall increase in pulp quality.

1. Introduction

Dissolving pulp is a low-yield chemical pulp (30-35%) with a high cellulose (95-98%) and relatively low hemicellulose (1-10%) and lignin (<0.05%) content (Christov

73 *et al*., 1998). It is produced by either prehydrolysis-kraft or acid sulphite pulping
74 (Bierman, 1993). Due to decreased demand for paper and increasing applications for
75 dissolving pulp, many paper mills are converting to dissolving pulp mills. Sappi-
76 Chemical Cellulose (Umkomaas, KZN), the world's largest producer of dissolving
77 pulp (>800 000 tonnes/year chemical cellulose), utilizes the acid sulphite process with
78 a cooking acid composed of free sulphur acid and sulphur acid bound as magnesium
79 bi-sulphite. As the cooking acid penetrates the wood, the lignin is degraded and
80 converted into a water-soluble substance that can be washed out. Sulphite pulp is
81 slightly brown and requires a chlorine-free bleaching step thereafter (Sappi-Chemical
82 Cellulose, 2007). Chemical cellulose is sold to manufacturers for a wide range of
83 consumer products, such as clothing, cellular phone screens, cellophane wrap for
84 sweets and flowers, pharmaceutical and household products, and makeup (Sappi,
85 2011).

86 In South Africa *Eucalyptus* forms more than 95% of wood material for pulping.
87 When the logs enter the mill, they are chipped and stored in piles up to 50 m high. A
88 recent review found that the origin of microbial communities in wood chip piles and
89 logs may differ as the microbial community composition differed in several reports.
90 (Noll and Jirjis, 2012). In addition, enhanced accessibility of substrates in and a
91 greater surface area of comminuted wood chips may explain community shifts and
92 extensive colonisation.

93 High temperatures and spontaneous combustion are reported as the cause of major
94 losses of chips (Ferrero et al., 2009; Fuller, 1985; Tansey, 1971) and are the result of
95 microbial fermentation (Li et al., 2006). During the first five to seven days of storage
96 the living cells remain viable and continue to respire resulting in heat generation, with
97 the highest temperatures reported at the centre of the pile (Fuller, 1985; Nurmi, 1999).

At temperatures of 60-70ºC the acetyl group attached to each cellulose molecule is cleaved forming acetic acid which causes the wood to darken and ultimately disintegrate as if burned (Fuller, 1985). The use of these wood chips in the 'cooking' process results in low yields and high pulpscreen rejects (Li et al., 2006).

Fuller (1985) created a protocol for chip pile management by identifying the causes and recommending ways to avoid deterioration. Pile height should be maintained below 15 m to prevent compaction and allow for heat release produced during microbial metabolism. Tractor spreading of recently chipped wood should be avoided, as this could create 'fines'. Sawdust/fines shavings should be avoided as they add to compaction and trapping of heat (Fuller, 1985; Hodland and Marques, 2003). Fuller has also recommended that wood species with different deterioration rates should be mixed as needed, so that chips that have a high deterioration rate are not clumped together in the pile creating a large zone with high temperatures (Fuller, 1985). To avoid spontaneous combustion or heating, material must not be damp and not stored in large volumes (Li et al., 2006). In addition to the above effects, wood chips could also undergo degradation by microbial enzyme action (Eriksson et al., 1990).

Cellulose degradation to glucose requires the synergistic action of three major cellulases; viz., endoglucanases, exoglucanases and β-glucosidases can be produced as discrete units or as multi-protein complexes (Lynd et al., 2002). Bacteria affect wood permeability and damage wood structure in synergistic action with soft-rot fungi which predisposes wood to fungal attack. Bacterial cellulolytic and pectinolytic enzyme systems play a significant part in structural changes in wood. Bacterial cellulases alter the permeability of wood by opening up the crystalline arrangement of the cellulose as a target for further diffusion of cellulolytic enzymes. White-rot and

soft-rot fungi cause rapid and widespread decay of all wood components by enzymatic degradation. Wood bleaching is also observed due to lignin removal by white-rot fungi. In the United States and Europe, a commercially available white mutant of *Ophiostoma piliferum* called Cartapip 97® is applied to pulpwood chips to prevent sapstain and minimize pitch (De Beer et al., 2003). Sithole et al. (2002) evaluated the effects of Cartapip™ on aspen chips in South Africa and reported higher strength properties of the pulp compared to untreated chips, after a three week period. An understanding of the natural wood chip microflora and the enzymes they produce may not only facilitate the development of a biological agent/treatment to assist the biopulping process but also provide a simple strategy to control pile degradation and ultimately pulp quality.

This study is the first direct comparison of the microflora indigenous to two *Eucalyptus* species. The effect of exposure to simulated weather conditions on microflora and their potential effect on the chemical properties of wood chips is also presented. Finally the study provides proof of concept that combining wood of different deterioration rates during storage can result in better quality pulp.

2. Materials and Methods

2.1 Wood Chip Collection

Wood chips were obtained from Sappi-Chemical Cellulose located in Umkomaas on the East Coast of KwaZulu-Natal. Log deliveries from different plantations along the south coast and inland areas enter the plant and are immediately processed and sent to the woodyard for chipping. After clearance of the conveyer belt, *E. dunnii* and *E. nitens* logs were selected and chipped. The wood chips were transported back to the lab and stored at 4°C (short term) and -20°C (long term).

148 **2.2 Simulation setup**

149 Triplicate chip piles (~ 3 kg each) of each of *E. dunnii*, *E. nitens* and a
150 combination (1:1) of these two species were exposed to simulated summer and winter
151 conditions in a Conviron Climatic Chamber (Microclima Series, Snijders Scientific,
152 Tilburg-Holland). Temperature and humidity settings for summer (25°C and 65%
153 humidity) and winter (16°C and 60% humidity) were determined from averaging
154 weather data received from the South African Weather Services.

155

156 **2.3 Sample Collection and Processing**

157 The simulation piles were sampled at time zero, two and four weeks of incubation
158 at three sampling points (section 1, 2 and 3; representing the top middle and bottom of
159 the pile, respectively). Samples were milled for chemical analyses and DNA
160 extraction. After completion of the simulation phases, the remaining chips in the piles
161 were dried at 50°C for one week and then pulped using the acid bi-sulphite method
162 (140°C, 850KPa) (Forestry and Forest Products Research Center, CSIR, Personal
163 Communication, 2010).

164

165 **2.4 Isolation of Bacteria and Fungi and Screening for Lignocellulolytic**
166 **Enzymes**

167 In order to isolate the predominant microorganisms indigenous to *E. dunnii* and *E.*
168 *nitens*, 5 g of wood chips for each of the wood species (Time zero samples) were
169 washed thoroughly with 5 ml phosphate buffer (pH 8) by vortexing for 5 min. The
170 washings were diluted and spread onto nutrient agar and potato dextrose agar (PDA)
171 and incubated at 37°C and 30°C for 4 and 6 days for bacteria and fungi, respectively.
172 Colonies were selected based on size, shape, pigmentation, margin, consistency and

elevation of the colony. Agar plates supplemented with either 1% (w/v) birchwood xylan (Sigma-Aldrich, USA), carboxymethylcellulose (Sigma-Aldrich, USA), or lignin (Sigma-Aldrich, USA) were used to screen for xylanase, cellulase and ligninase activity, respectively. Following incubation for 24 hours at 30°C and 37°C for fungi and bacteria respectively, plates were then stained with 0.1% Congo red for 30 minutes and destained with 1 M NaCl for 1 hour. The presence of "halos" were indicative of enzyme activity (Zhang et al., 2006).

2.5 DNA Isolation

DNA was extracted from 0.2 g milled chips using a kit as per manufacturer's specifications (Soil DNA Extraction Kit, Zymo Research). Genomic DNA was isolated from the pure isolates using the ZR Fungal/Bacterial DNA Kit (Zymo Research), as per manufacturer's instructions.

2.6 PCR

Ribosomal genes were amplified from microbial genomic DNA from milled chips and purified cultures. 16S and the ITS region of the 18S rRNA genes were amplified using universal primer sets: 63F/1387R (Marchesi et al., 1998) and ITS5F/ITS4R (White et al., 1990), respectively. Each amplification reaction (50 µl) contained 1.25 mM $MgCl_2$, 0.125 µM forward and reverse primers, 0.2 mM deoxynucleoside triphosphate (dNTPs), 0.25 U SuperTherm *Taq* DNA polymerase (Southern Cross Biotech), and 20-200 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

198 min and a final extension at 72°C for 5 min, whereas 18S rRNA amplification
199 conditions were: initial denaturation at 95°C for 2 min, 25 cycles of 95°C for 30 sec,
200 53°C for 45 sec, 72°C for 1 min and a final extension at 72°C for 8 min. The
201 amplicons were analyzed by electrophoresis on 1% agarose (SeaKem) gels in 1× TAE
202 running buffer at 90 V for 45 min. After electrophoresis, the gels were stained in 0.5
203 µg/ml ethidium bromide and visualized using the Chemi-Genius 2 BioImaging
204 System (Syngene). Following PCR, the amplicons were sequenced (Inqaba Biotech,
205 Pretoria). The sequence data received was edited and entered in the BLAST algorithm
206 (Altshul et al., 1990) for identification of microorganisms.
207 Upon confirmation of 16S and 18S amplicons, products were purified using a
208 GeneJET™ PCR purification kit (Fermentas) and re-amplified in a touchdown thermal
209 profile program using nested PCR and primers with GC clamps. PCR for 16S rRNA
210 genes were initially performed using two primer sets: 338F-GC with a GC-clamp: 5´-
211 CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´ and 518R
212 (237-bp fragment) (Handschur et al., 2005); 933F-GC with a GC-clamp: 5´-
213 CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´ and 1387R
214 (500-bp fragment) (Ji et al., 2004). The primer sets NS26/518R-GC (316-bp
215 fragment) and EF4F/518R-GC (426-bp fragment) were also tested for amplification of
216 the 18S rRNA genes, with the same GC-clamp: 5´-
217 CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´
218 (Schabereiter-Gurtner et al., 2001). The composition of the reaction mixtures were the
219 same as that used for the first PCR. For amplification of 16S rRNA, PCR conditions
220 were as follows: initial denaturation at 95°C for 5 min, 30 cycles of denaturation
221 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).

2.7 DGGE

DGGE was performed using the D-Code Universal Mutation Detection System (BioRad), and an optimized method (Govender et al. 2013) modified from Muyzer et al. (1998). PCR samples were loaded onto vertical perpendicular polyacrylamide gels (6% acrylamidebisacrylamide [37.5:1]) in 1× 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) 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). A marker consisting of known microbial species which were isolated, cultured and identified together with bands that were excised, re-amplified and sequenced from other DGGE gels were used to infer identities in the environmental samples without having to excise each band for sequencing.

2.8 Identification of microorganisms using culturing techniques and DGGE

Genomic DNA was extracted from the milled wood chips using a Soil DNA Extraction kit (Zymo Research), with the following modifications: 0.2 g of sample in

1200 µl of lysis buffer. Succeeding PCR and DGGE analysis, DNA was eluted from excised bands and re-amplified using the same primer set excluding the GC-clamp. Genomic DNA was isolated from the pure isolates and amplified using universal bacterial and fungal primers 16S and 18S, respectively. PCR products were sequenced (Inqaba Biotech, Pretoria). Sequence data were edited and submitted to Genbank for comparison to sequences in the database for identification of genus and species (Altschul et al. 1990).

2.9 Chemical Analyses

Chemical characteristics of the wood chips were evaluated by conducting extractive analyses and these included: (i) Soxhlet extraction method for determination of solvent (water insoluble) extractives, determination of "hot water" (water soluble) extractives (waxes, fats, some resins, photosterols, and non-volatile hydrocarbons, low molecular weight carbohydrates, salts, and other water soluble substances); (ii) Gas Chromatography – Mass Spectrometry (GCMS) for the characterisation of lignin; (iii) Near Infra-red reflectance analyser (NIRA) for the rapid quantification of wood chemical components; and (iv) 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). Pulp quality was determined by assessing viscosity (Tappi T203 om-94; Grix, 2002), final pulp yield, kappa number, alpha cellulose, S8, S10 (Tappi T235-OM60; Grix 2002), and hemicelluloses (Forestry and Forest Products Research Center, CSIR, Personal Communication, 2010).

2.10 Statistical Analyses

Data was analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. Probability values less than 5% ($p<0.05$) were considered significant.

3. Results

3.1 Isolation of Bacteria and Fungi

Five and nine fungal and eight and eight bacterial isolates were identified from *E. nitens* and *E. dunnii*, respectively (Table 1 and 3) using traditional culturing methods. Three *Bacillus* spp. (seven isolates), two *Curtobacterium* spp., *Pantoea* spp., and *Mucilaginibacter* sp. were identified. *Bacillus* spp. were predominant (44% of bacteria isolated) in both *E. dunnii* and *E. nitens*. *Penicillium* spp. were predominant (80 and 56% of the fungi isolated) in *E. nitens* and *E. dunnii*, respectively.

3.2 Analysis of Microbial populations

It is clearly evident from the DGGE profiles that *E. nitens* (four bacterial isolates at time 0) has a more diverse bacterial and fungal population than *E. dunnii* (nine at time 0). There was also an increase in microbial diversity and abundance from the freshly chipped samples (time zero) to the winter and summer simulations. Sixteen and seven more bacterial species were identified from *E. nitens* and *E. dunnii*, respectively, using DGGE (Figure 1, Table 2). Fungal profiles revealed eight and four fungal species from *E. nitens* and *E. dunnii*, respectively, which were not identified using the culturing technique (Figure 2, Table 4). Variable patterns were obtained based on *Eucalyptus* sp., season and location in pile as evidenced by increasing/decreasing number of bands and/or intensity of bands correlating to

297 appearance/disappearance of certain species and/or increasing/decreasing
298 abundance/dominance. *Pseudomonas* sp., *Clavibacter* sp., *Streptomyces* sp.,
299 *Shewanella* sp., *Mycrobacterium* sp. were unique to *E. nitens* and a few were also
300 present in the combination. *Bacillus thuringiensis* was prevalent in *E. nitens*, *E. dunnii*
301 and combination piles. Its abundance, however, varied amongst the samples - more
302 abundant in *E. dunnii* than *E. nitens*. In the combination pile *B. thuringiensis* was
303 more abundant in summer than winter. *Bacillus* sp. R-43588 appeared to be
304 predominant in *E. nitens* (winter), less abundant in the combination pile and absent in
305 *E. dunnii*. *Bacillus* sp. NBSL38 was present in *E. dunnii* and *E. nitens* during both
306 season simulations. Variations in population density of *Gemella sanguinis*,
307 *Modestobacter* sp and *Pantoea agglomerans* were apparent across *Eucalyptus* species,
308 season and section sampled. Overall, visual analysis indicated the greatest diversity
309 and abundance of microorganisms in *E. nitens*. But when combined with *E. dunnii*,
310 some diversity was lost and a few unique bands gained.
311
312 Fewer fungal species were observed compared to bacterial profiles for each of the
313 wood species and the combination pile. *Penicillium decumbens* s1821 was present
314 amongst most of the *E. dunnii* and *E. nitens* samples. However, this species was not
315 detected in the combination pile. Similar multiple bands representing *Mytilinidion*
316 *mytilinellum*, *Normandia pulchella*, *Teratosphaeria secundaria* and *Ascomycete* sp.
317 were observed throughout the winter and summer simulation of *E. nitens* and the
318 combination pile, but with varying intensity. *Lodderomyces elongisporus* and *P.*
319 *decumbens* appeared to be predominant amongst the *E. nitens* and combination pile
320 samples. *Scutellospora reticulata* was unique to *E. nitens*.
321

3.3 Screening for enzymes

Microorganisms were screened for their ability to produce cellulase, ligninase and xylanase. The presence of dual enzyme activity and all three enzymes appeared to be more ubiquitous than individual enzyme activities (Table 1 and 3). Only 12.5% and 6.25% of the bacterial isolates displayed either cellulase or xylanase activity, respectively. Fourteen percent of the fungal isolates displayed xylanase exclusively, whilst none were capable of producing cellulase. Dual cellulase and xylanase activity was observed in 6.25% and 42.9% of the bacterial and fungal isolates, respectively. The activity of all three enzymes was noted for 6.25% of the bacteria and 28.6% of the fungi. All ligninase producers were isolated from *E. dunnii*, and 86% of the fungal isolates were identified as xylanase-producers. Fungal isolates from *E. nitens* displayed the highest levels of xylanase activity, and in particular *P. spinulosum* (*E. nitens* 8) had high xylanase and low ligninase and cellulase activity.

3.4 Chemical Analyses

Comparison of time zero samples for *E. dunnii* and *E. nitens* provides a baseline difference in the properties of the two wood species. A trend between the hot water extractives, cellulose, glucose and acid insoluble lignin was evident. Cellulose and glucose levels were significantly higher in *E. nitens* than *E. dunnii*. with significantly higher cellulose content for winter than summer ($p<0.05$) (Figure 5). The level of hot water extractives content was significantly higher in *E. dunnii* ($p<0.05$). Significantly lower hot water extractive content and higher acid insoluble lignin were noted in summer for *E. dunnii*, *E. nitens* and the combination wood.

Pulp viscosity (degree of polymerization of the cellulose fibres) reflects the relative integrity of the cellulose fibres after pulping. No significant differences in pulp viscosity were observed after summer and winter simulations for all three experiments, however significant differences were observed for time zero and after summer and winter simulations (Figure 6). The total cellulose content of the wood chips ranged between 40-45%. Standardized pulping methods recovered approximately 95% alpha cellulose resulting in pulp yields between 46 and 55%. Highest pulp yields and greater viscosity were observed in the summer simulation of *E. nitens*, followed by the combination piles and *E. dunnii* ($p<0.05$). Pulp yields after the winter simulation were similar for *E. dunnii* and the combination but higher for *E. nitens*.

The degraded cellulose in the pulp is represented by the subtraction of S18 from S10. Lowest cellulose degradation was noted for the summer and winter simulations for *E. nitens*, followed by the combination pile and *E. dunnii*. These trends corroborate the viscosity values for the pulp. Total lignin and klason lignin of the final pulp were higher for the winter simulation of *E. dunnii* and *E. nitens*, however, in the combination pile levels were similar for both seasons (Figure 7). Overall, *E. nitens* yielded the highest pulp yields and viscosity and *E. dunnii* lowest pulp yields and viscosity. The combination piles on the other hand produced higher pulp yields (0.9%) and lower cellulose degradation for the summer simulation and higher α cellulose levels in the winter simulation compared to averaged data for the two *Eucalyptus* species.

4. Discussion

This investigation provides proof of the concept that combination of wood species with different deterioration rates as proposed by Fuller (1985) results in an overall increase in the pulp yields, especially during summer simulation. The increase in yield of 0.9% obtained is significant since even a 0.5% increase is regarded as desirable by commercial mills (personal communication, Operations Manager, Sappi) Other positive effects in the combination piles were evident in during winter (lower lignin, higher α-cellulose) and in summer (higher yield and lower degraded cellulose). The negative effects in summer were lower viscosity and α-cellulose and winter higher cellulose degradation and lower viscosity. Hot water extractives in the combination piles were significantly lower than for either *Eucalyptus* species. As this is a sought after property (Gutierrez et al., 1998) in the pulping industry (results in decreased pitch deposits), this merits further investigation to elucidate which microorganisms or enzyme sets are responsible for the effect. Careful thought needs to be put into which species are combined as we observed variable results depending on the season. Indigenous microflora of each wood species may be one of the contributing factors to poor/good pulp quality, as significant correlations were made between the enzymes produced by microorganisms and pulp quality and yields.

The inherent properties of wood chips from different *Eucalyptus* species is reflected in the pulp produced and conforms with data presented by Ndukwe et al. (2009). *E. nitens* pulp yields of were lower in summer, possibly due to the higher intensity of fungal populations. Low viscosity values for *E. dunnii* relative to the time zero sample may be linked to the high number of cellulase producers isolated from this species that could be responsible for the degradation of cellulose. Viscosity

values for the combination pile were unexpectedly low and may be attributed to endoglucanase activity, i.e., random cleavage of cellulose chains with lower degrees of polymerization and therefore lower viscosity.

The retention of cellulose fibres is vital for producing strong pulp, thus the negative effect of cellulases on pulp quality needs to be minimized. Eighty six percent of the fungal isolates displayed varying levels of cellulase activity only or in combination with other enzymes, which is an undesirable characteristic. On the other hand, 43% and 86% of the fungal isolates were identified as ligninase and xylanase producers, respectively. The presence of xylanases may assist in the pulp beaching process, as it removes xylan which allows for easier bleaching of the cellulose fibres thus utilizing less bleach and energy (Garg et al., 2011; Kaur et al., 2010). The degradation of xylan fibres also improves the removal of lignin by other microorganisms, which is important since lignin also forms an obstruction during pulp bleaching (Subramaniyan and Prema, 2002).

Correlations can be made between microbial population, seasons, enzyme activity, and changes in chemistry, pulp quality and yield. Positive effects were seen with a decrease in lipophilic extractives, which aids in the reduction of pitch deposit formation. The negative effects of cellulose activity on the pulp yield were also evident. Construction of wood chip piles needs to be planned as wood species and enzymes produced by indigenous microflora were shown to have a potential impact on the final pulp product. To gain a better understanding of microflora as a variable in pulping, all effects need to be studied further to evaluate the contributing factors that lead to the production of poor quality pulp and low pulp yields.

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References

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology **215:**403 – 410

Bierman, C.J. 1993. Essentials of Pulping and Papermaking, **p.** 72 – 100. Academic Press, New York

De Beer, Z. W., B. D. Wingfield, and M. J. Wingfield. 2003. The occurrence of *Ophiostoma piliferum*-like fungi on pulpwood chips and other wood sources in South Africa. *South African Journal of Science* **99:**34 – 36

Eriksson, K. E. L., R. A. Blanchette, and P. Ander. 1990. Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, Berlin, Germany

Ferrero, F., C. Lohrer, B. M. Schmidt, M. Noll, and M. Malow. 2009. A mathematical model to predict the heating-up of large scale wood piles. *Journal of Loss Prevention in the Process Industries* **22:**439 – 448

Fuller, W. S. 1985. Chip pile storage – a review of practices to avoid deterioration and economic losses. *Tappi Journal* **68:**48 – 52

Garg, G., S. S. Dhiman, R. Mahajan, A. Kaur, and J. Sharma. 2011. Bleach-boosting effect of crude xylanase from *Bacillus stearothermophilus* SDX on wheat straw pulp. *New Biotechnology* **28:**58 – 64

452 Govender, L., T. Bush, R. Govinden. 2013. Method optimization for DGGE analysis
453 of microflora from industrial wood chips intended for pulping. *Online International*
454 *Journal of Microbiology Research*, In press
455
456 Handschur, M., G. Pinar, B. Gallist, W. Lubitz, and A. G. Haslberger. 2005. Culture
457 free DGGE and cloning based monitoring of changes in bacterial communities of
458 salad due to processing. *Food and Chemical Toxicology* **43:**1595 – 1605
459
460 Hodland, W. and M. Marques. 2003. Physical, biological and chemical processes
461 during storage and spontaneous combustion of waste fuel. *Resources, Conservation*
462 *and Recycling* **40:**53 – 69
463
464 Ji, N., B. Peng, G. Wang, S. Wang, and X. Peng. 2004. Universal primer PCR with
465 DGGE for rapid detection of bacterial pathogens. *Journal of Microbiological Methods*
466 **57:**409 – 413
467
468 Kaur, A., R. Mahajan, A. Singh, G. Garg, and J. Sharma. 2010. Application of
469 cellulase-free xylano-pectinolytic enzymes from the same bacterial isolate in
470 biobleaching of kraft pulp. *Bioresource Technology* **101:**9150 – 9155
471
472 Li X, H. Koseki, and M. Momota. 2006. Evaluation of danger from fermentation-
473 induced spontaneous ignition of wood chips. *Journal of Hazardous Materials* **A135:**
474 15-20
475
476 Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial
477 cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular*
478 *Biology Reviews* **66:**506 – 577
479
480 Marchesi, J. R., T. Sato, A. J. Weightman, J. C. Fry, and S. J. Hiom. 1998 Design and
481 evaluation of useful bacterial-specific PCR primers that amplify genes coding for
482 bacterial 16s rRNA. *Applied and Environmental Microbiology* **64:**795 – 799
483

Muyzer, G. and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73:**127 – 141

Ndukwe, N. A., F. O. Jenmi, W. O. Okiei, and B. I. Alo. 2009. Comparative study of percentage yield of pulp from various Nigerian wood species using the kraft process. *African Journal of Environmental Science and Technology* **3:**21 – 25

Noll, M., and R. Jirjis. 2012. Microbial communities in large-scale wood piles and their effects on wood quality and the environment. *Applied Microbiology Biotechnology* **95:**551 – 563

Nurmi, J. 1999. The storage of logging residue for fuel. *Biomass and Bioenergy* **17:**41 – 47

Sappi. 2011. Available at; http://www.sappi.com/regions/sa/service/mediadownloads/Pages/default.aspx. Accessed: 7/05/2012

Sappi-Chemical Cellulose (2007) The Paper Making Process: From wood to coated paper. Sappi Europe SA, Accessed: June 2010. Available at http://www.google.co.za/url?sa=t&rct=j&q=the%20paper%20making%20process%3A%20from%20wood%20to%20coated%20paper.%20sappi%20europe%20sa&source=web&cd=1&cad=rja&ved=0CC0QFjAA&url=http%3A%2F%2Fwww.na.sappi.com%2Fdocuments%2F10165%2F13508%2FThe%2BPapermaking%2BProcess.pdf&ei=YldJUZ7uDtO5hAep_oHwBg&usg=AFQjCNEAVE4i5vYUqXZZw8CtGBYFITjf3g

Schabereiter-Gurtner, C., G. Piñar, W. Lubitz, and S. Rölleke. 2001. Analysis of fungal communities on historical church window glass by denaturing gradient gel electrophoresis and phylogenetic 18S rDNA sequences analysis. *Journal of Microbiological Methods* **47:**345 – 354

Sithole, B., M.-J. Rocheleau, R. Berlyn, C. Heitner, and L. Allen. 2002. Is fungal treatment of Aspen chips beneficial for CTMP? Technical Association of the Pulp and

Paper Industry of Southern Africa, African Pulp and Paper Week, International Convention Centre, Durban, 8 – 11 October

Subramaniyan, S. and P. Prema. 2002. Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology and Application. *Critical Reviews in Biotechnology* **22:**33 – 46

Tansey, M. R. 1971. Isolation of Thermophilic Fungi from Self-Heated, Industrial Wood Chip Piles. *Mycologia* **63:**537 – 547

TAPPI, 1996. Test methods T249 cm-85: Carbohydrate anaylsis; T222 om-88: Acid insoluble lignin of wood and pulp; T204 om-88: Solvent extractives. Tappi Test Methods (1996-1997), Tappi Press, Atlanta, USA

Wallis, A. F. A., R. H. Wearne, and P.J. Wright. 1996. Chemical analysis of polysaccharides in plantation eucalypt woods and pulps. *Appita Journal* **49:**258 – 262

White, T. J., T. Burns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, **p.** 315-322. *In*: M. A. Innes, D. H. Gelfand, J. J. Sninsky, T. J. White, (ed.), PCR Protocols. Academic Press. San Diego, CA

Wright, P. J. and A. F. A Wallis. 1996. Rapid determination of carbohydrates in Hardwoods. *Holzforschung* **50:**518 – 524

Zhang, Y.-H. P., M. E. Himmel, and J. R. Mielenz. 2006. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances* **24:**452 – 481

Table 1: Identification and screening of bacterial isolates from *E. dunnii* and *E. nitens*

Pure Isolates	Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Cellulase	Ligninase	Xylanase
E. nitens 1	*Bacillus cereus*	Firmicutes	JF758862.1	98	++	-	+
E. nitens 2	*Pantoea* sp.	Proteobacteria	JN853250.1	76	-	-	-
E. nitens 3	*Curtobacterium sp.*	Actinobacteria	HQ219967.1	97	-	-	-
E. nitens 4	*Bacillus cereus*	Firmicutes	JQ308572.1	98	-	-	-
E. nitens 5	*Bacillus cereus*	Firmicutes	EU621383.1	97	-	-	-
E. nitens 6	*Bacillus* sp.	Firmicutes	EU162013.1	98	-	-	-
E. nitens 7	*Bacillus thuringiensis*	Firmicutes	FN667913.1	97	-	-	-
E. nitens 8	*Unidentified*	-	-	-	+	-	-
E. dunnii 1	*Mucilaginibacter sp.*	Bacteroidetes	JF999998.1	84	-	-	-
E. dunnii 2	*Unidentified*	-	-	-	-	-	-
E. dunnii 3	*Curtobacterium flaccumfaciens*	Actinobacteria	HE613377.1	98	-	-	-
E. dunnii 4	*Unidentified*	-	-	-	-	-	+
E. dunnii 5	*Pantoea vagans*	Proteobacteria	CP002206.1	99	-	-	-
E. dunnii 6	*Unidentified*	-	-	-	+	-	-
E. dunnii 7	*Bacillus thuringiensis*	Firmicutes	FN667913.1	99	++	+	+
E. dunnii 8	*Unidentified*	-	-	-	-	-	-

Key: + Slight halo ++ Medium halo +++ Large halo

565 **Table 2:** Bacteria identified by sequencing of 16S rRNA amplicons of excised bands from PCR-DGGE gels

	Time Zero					Summer					Winter				
	Bacterial Species	**Phyla**	**Best Match database (Gene Bank Accession No.)**	**Similarity (%)**	**Accession Number**	**Bacterial Species**	**Phyla**	**Best Match database (Gene Bank Accession No.)**	**Similarity (%)**	**Accession Number**	**Bacterial Species**	**Phyla**	**Best Match database (Gene Bank Accession No.)**	**Similarity (%)**	**Accession Number**
E. dunnii	*Arthrobacter globiformis*	a	JF439620.1	98	KC493062	D1					D1				
	Bacillus thuringiensis	b	HQ432813.1	99	KC020161	Uncultured bacterium	-	HM658573.1	95	KC493045	Uncultured bacterium	-	HM658573.1	95	KC493045
	Gemella sanguinis	b	GU426152.1	93	*	*Bacillus thuringiensis*	b	FJ755917.1	96	KC493046	*Bacillus thuringiensis*	b	FJ755917.1	96	KC493046
	Bacillus sp. [2]	b	HM197763.1	97	KC493065	*Bacillus cereus*	b	AM397642.1	100	KC493049	*Pseudomonas* sp. [1]	c	FM161626.1	100	KC493047
						Cellulomonas sp.	a	HF566196.1	98	KC493051	*Arthrobacter globiformis*	a	JF439620.1	98	KC493062
						Bacillus thuringiensis	b	HQ432813.1	99	KC020161	*Bacillus thuringiensis*	b	HQ432813.1	99	KC020161
						Bacillus sp. [1]	b	JX317704.1	100	KC493053	*Gemella sanguinis*	b	GU426152.1	93	*
						Erwinia sp.	c	FM161470.1	100	KC493056	*Bacillus* sp. [2]	b	HM197763.1	97	KC493065
						Klebsiella sp.	c	AY880196.1	93	KC020162					
						Anoxygenic photosynthetic bacterium	-	FJ036922.1	87	KC493059					
						Methylobacterium sp.	c	GQ342553.1	100	KC493060					
						Gemella sanguinis	b	GU426152.1	93	*					
						Uncultured *Geobacteraceae*	c	EF658390.1	95	KC493061					
						Arthrobacter globiformis	a	JF439620.1	98	KC493062					
						Bacillus sp. [2]	b	HM197763.1	97	KC493065					
E. nitens	D1					Uncultured bacterium	-	HM658573.1	95	KC493045	D1				
	Uncultured bacterium	-	HM658573.1	95	KC493045	*Bacillus thuringiensis*	b	FJ755917.1	96	KC493046	Uncultured bacterium	-	HM658573.1	95	KC493045
	Bacillus thuringiensis	b	FJ755917.1	96	KC493046	*Pseudomonas* sp. [1]	c	FM161626.1	100	KC493047	*Bacillus thuringiensis*	b	FJ755917.1	96	KC493046
	Arthrobacter globiformis	a	JF439620.1	98	KC493062	*Pseudomonas* sp. [2]	c	FR775123.1	97	KC493048	*Pseudomonas* sp. [1]	c	FM161626.1	100	KC493047

E. nitens	*Streptomyces* sp.	a	GU132502.1	99	KC493050	*Bacillus cereus*	b	FR695425.1	97	*	*Bacillus cereus*	b	FR695425.1	97	*
	Cellulomonas sp.	a	HF566196.1	98	KC493051	*Clavibacter* sp.	a	JX949715.1	97	KC493058	*Clavibacter* sp.	a	JX949715.1	97	KC493058
	Bacillus thuringiensis	b	HQ432813.1	99	KC020161	*Arthrobacter globiformis*	a	JF439620.1	98	KC493062	*Arthrobacter globiformis*	a	JF439620.1	98	KC493062
	Pseudomonas sp. [3]	c	DQ282193.1	97	KC493054	*Streptomyces* sp.	a	GU132502.1	99	KC493050	*Streptomyces* sp.	a	GU132502.1	99	KC493050
	Shewanella sp.	c	AY536556.1	90	KC493055	*Cellulomonas* sp.	a	HF566196.1	98	KC493051	*Cellulomonas* sp.	a	HF566196.1	98	KC493051
	Gemella sanguinis	b	GU426152.1	93	*	*Pantoea* sp.	c	AB478135.1	98	KC493052	*Bacillus thuringiensis*	b	HQ432813.1	99	KC020161
	Bacillus sp. [2]	b	HM197763.1	97	KC493065	*Bacillus* sp. [1]	b	JX317704.1	100	KC493053	*Bacillus* sp. [1]	b	JX317704.1	100	KC493053
						Pseudomonas sp. [3]	c	DQ282193.1	97	KC493054	*Pseudomonas* sp. [3]	c	DQ282193.1	97	KC493054
						Shewanella sp.	c	AY536556.1	90	KC493055	*Shewanella* sp.	c	AY536556.1	90	KC493055
						Erwinia sp.	c	FM161470.1	100	KC493056	*Erwinia* sp.	c	FM161470.1	100	KC493056
						Pantoea agglomerans	c	HE647624.1	97	KC493057	*Pantoea agglomerans*	c	HE647624.1	97	KC493057
						Klebsiella sp.	c	AY880196.1	93	KC020162	*Klebsiella* sp.	c	AY880196.1	93	KC020162
						Anoxygenic photosynthetic bacterium	-	FJ036922.1	87	KC493059	Anoxygenic photosynthetic bacterium	-	FJ036922.1	87	KC493059
						Methylobacterium sp.	c	GQ342553.1	100	KC493060	*Gemella sanguinis*	b	GU426152.1	93	*
						Gemella sanguinis	b	GU426152.1	93	*	*Bacillus* sp. [2]	b	HM197763.1	97	KC493065
						Uncultured *Geobacteraceae*	c	EF658390.1	95	KC493061					
						Uncultured Mycobacterium sp.	a	GU556378.1	95	KC493064					
						Bacillus sp. [2]	b	HM197763.1	97	KC493065					
Combination	D1					D1					D1				
	Uncultured bacterium	-	HM658573.1	95	KC493045	Uncultured bacterium	-	HM658573.1	95	KC493045	Uncultured bacterium	-	HM658573.1	95	KC493045
	Bacillus thuringiensis	b	FJ755917.1	96	KC493046	*Bacillus thuringiensis*	b	FJ755917.1	96	KC493046	*Bacillus thuringiensis*	b	FJ755917.1	96	KC493046
	Clavibacter sp.	a	JX949715.1	97	KC493058	*Clavibacter* sp.	a	JX949715.1	97	KC493058	*Clavibacter* sp.	a	JX949715.1	97	KC493058
	Bacillus sp. [1]	b	JX317704.1	100	KC493053	*Cellulomonas* sp.	a	HF566196.1	98	KC493051	*Cellulomonas* sp.	a	HF566196.1	98	KC493051

	Klebsiella sp.	c	AY880196.1	93	KC020162	*Pantoea* sp.	c	AB478135.1	98	KC493052	*Bacillus thuringiensis*	b	HQ432813.1	99	KC020161
	Arthrobacter globiformis	a	JF439620.1	98	KC493062	*Bacillus thuringiensis*	b	HQ432813.1	99	KC020161	*Bacillus* sp. [1]	b	JX317704.1	100	KC493053
	Modestobacter sp.	a	JX982719.1	89	KC493063	*Bacillus* sp. [1]	b	JX317704.1	100	KC493053	*Erwinia* sp.	c	FM161470.1	100	KC493056
	Uncultured Mycobacterium sp.	a	GU556378.1	95	KC493064	*Pantoea agglomerans*	c	HE647624.1	97	KC493057	*Pantoea agglomerans*	c	HE647624.1	97	KC493057
						Klebsiella sp.	c	AY880196.1	93	KC020162	*Klebsiella* sp.	c	AY880196.1	93	KC020162
						Anoxygenic photosynthetic bacterium	-	FJ036922.1	87	KC493059	Anoxygenic photosynthetic bacterium	-	FJ036922.1	87	KC493059
						Methylobacterium sp.	c	GQ342553.1	100	KC493060	*Gemella sanguinis*	b	GU426152.1	93	*
						Gemella sanguinis	b	GU426152.1	93	*	*Arthrobacter globiformis*	a	JF439620.1	98	KC493062
						Modestobacter sp.	a	JX982719.1	89	KC493063	Uncultured Mycobacterium sp.	a	GU556378.1	95	KC493064
						Uncultured Mycobacterium sp.	a	GU556378.1	95	KC493064					
						Bacillus sp. [2]	b	HM197763.1	97	KC493065					

a= Actinobacteria b= Firmicutes c= Proteobacteria

* Accession numbers could not be assigned to excised bands that were less than 150 bp after sequencing.

578 **Table 3:** Identification and screening of fungal isolates from *E. dunnii* and *E. nitens*
579

Pure Isolates	Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Cellulase	Ligninase	Xylanase
E. nitens 1	*Penicillium adametzioides*	Ascomycota	DQ681325.1	99	+	-	++
E. nitens 2	*Aspergillus fumigatus*	Ascomycota	GU566217.1	98	+	-	+++
E. nitens 3	*Penicillium thomii*	Ascomycota	DQ132815.1	99	+	-	+++
E. nitens 4	*Penicillium spinulosum*	Ascomycota	HQ608158.1	100	+	-	+++
E. nitens 10	*Penicillium glabrum*	Ascomycota	GU565126.1	99	-	-	+
E. dunnii 1	*Penicillium spinulosum*	Ascomycota	HQ608085.1	100	+	++	++
E. dunnii 2	*Aspergillus fumigatus*	Ascomycota	HQ285617.1	94	+	-	+++
E. dunnii 3	*Unidentified*	-	-	-	+	-	+
E. dunnii 7	*Aspergillus fumigatus*	Ascomycota	GU566217.1	100	++	++	-
E. dunnii 8	*Penicillium adametzioides*	Ascomycota	DQ681325.1	99	++	++	+
E. dunnii 11	*Penicillium roqueforti*	Ascomycota	AB479313.1	100	-	-	+
E. dunnii 12	*Aspergillus fumigatus*	Ascomycota	GU992275.1	93	+	++	+
E. dunnii 13	*Penicillium commune*	Ascomycota	EU030337.1	98	+	+	-
E. dunnii 14	*Penicillium spinulosum*	Ascomycota	GU566247.1	99	+	+	++

580
581 **Key:** + Slight halo ++ Medium halo +++ Large halo
582
583

584 **Table 4:** Fungi identified by sequencing of 18S rRNA ITS amplicons of excised bands from PCR-DGGE gels

	Time Zero					Summer					Winter				
	Fungal Species	**Phyla**	**Best Match database (Gene Bank Accession No.)**	**Similarity (%)**	**Accession Number**	**Fungal Species**	**Phyla**	**Best Match database (Gene Bank Accession No.)**	**Similarity (%)**	**Accession Number**	**Fungal Species**	**Phyla**	**Best Match database (Gene Bank Accession No.)**	**Similarity (%)**	**Accession Number**
E. dunnii	Uncultured soil fungus	-	AY163435.1	98	KC493032	Uncultured soil fungus	-	AY163435.1	98	KC493032	Uncultured soil fungus	-	AY163435.1	98	KC493032
	Penicillium decumbens	a	HQ871900.1	99	KC493034	Uncultured fungus clone	-	EU696205.1	99	KC493033	Uncultured fungus clone	-	EU696205.1	99	KC493033
						Penicillium decumbens	a	HQ871900.1	99	KC493034	*Penicillium decumbens*	a	HQ871900.1	99	KC493034
						Aspergillus nomius	a	JQ045856.1	98	KC493035	*Aspergillus nomius*	a	JQ045856.1	98	KC493035
E. nitens	Uncultured fungus clone	-	EU696205.1	99	KC493033	Uncultured fungus clone	-	EU696205.1	99	KC493033	Uncultured fungus clone	-	EU696205.1	99	KC493033
	Penicillium decumbens	a	HQ871900.1	99	KC493034	*Penicillium decumbens*	a	HQ871900.1	99	KC493034	*Penicillium decumbens*	a	HQ871900.1	99	KC493034
	Lodderomyces elongisporus	a	EF120591.1	98	KC493036	*Lodderomyces elongisporus*	a	EF120591.1	98	KC493036	*Lodderomyces elongisporus*	a	EF120591.1	98	KC493036
	Penicillium decumbens	a	HQ455812.1	99	KC493037	*Penicillium decumbens*	a	HQ455812.1	99	KC493037	*Penicillium decumbens*	a	HQ455812.1	99	KC493037
	Mytilinidion mytilinellum	a	HM163570.1	99	KC493038	*Mytilinidion mytilinellum*	a	HM163570.1	99	KC493038	*Mytilinidion mytilinellum*	a	HM163570.1	99	KC493038
	Normandina pulchella	a	GU121580.1	97	KC493039	*Normandina pulchella*	a	GU121580.1	97	KC493039	*Normandina pulchella*	a	GU121580.1	97	KC493039
	Symbiotaphrina buchneri	a	AY227716.1	99	KC493040	*Symbiotaphrina buchneri*	a	AY227716.1	99	KC493040	*Symbiotaphrina buchneri*	a	AY227716.1	99	KC493040
	Ascomycete sp.	a	EU484181.1	99	KC493041	Ascomycete sp.	a	EU484181.1	99	KC493041	Ascomycete sp.	a	EU484181.1	99	KC493041
	Uncultured *Paecilomyces* sp.	a	KC020150.1	93	KC493042	Uncultured *Paecilomyces* sp.	a	KC020150.1	93	KC493042	Uncultured *Paecilomyces* sp.	a	KC020150.1	93	KC493042
						Uncultured eukaryote	-	HQ999349.1	90	KC493043	Uncultured eukaryote	-	HQ999349.1	90	KC493043
Combination	*Penicillium decumbens*	a	HQ871900.1	99	KC493034	*Lodderomyces elongisporus*	a	EF120591.1	98	KC493036	*Lodderomyces elongisporus*	a	EF120591.1	98	KC493036
	Penicillium decumbens	a	HQ455812.1	99	KC493037	*Penicillium decumbens*	a	HQ455812.1	99	KC493037	*Penicillium decumbens*	a	HQ455812.1	99	KC493037
	Normandina pulchella	a	GU121580.1	97	KC493039	*Mytilinidion mytilinellum*	a	HM163570.1	99	KC493038	*Mytilinidion mytilinellum*	a	HM163570.1	99	KC493038
	Symbiotaphrina buchneri	a	AY227716.1	99	KC493040	*Normandina pulchella*	a	GU121580.1	97	KC493039	*Normandina pulchella*	a	GU121580.1	97	KC493039

	Ascomycete sp.	a	EU484181.1	99	KC493041	*Symbiotaphrina buchneri*	a	AY227716.1	99	KC493040	*Symbiotaphrina buchneri*	a	AY227716.1	99	KC493040
						Ascomycete sp.	a	EU484181.1	99	KC493041	Ascomycete sp.	a	EU484181.1	99	KC493041
						Dacrymyces variisporus	b	AB712512.1	91	KC493044	*Dacrymyces variisporus*	b	AB712512.1	91	KC493044

a= Ascomycota
b= Basidiomycota

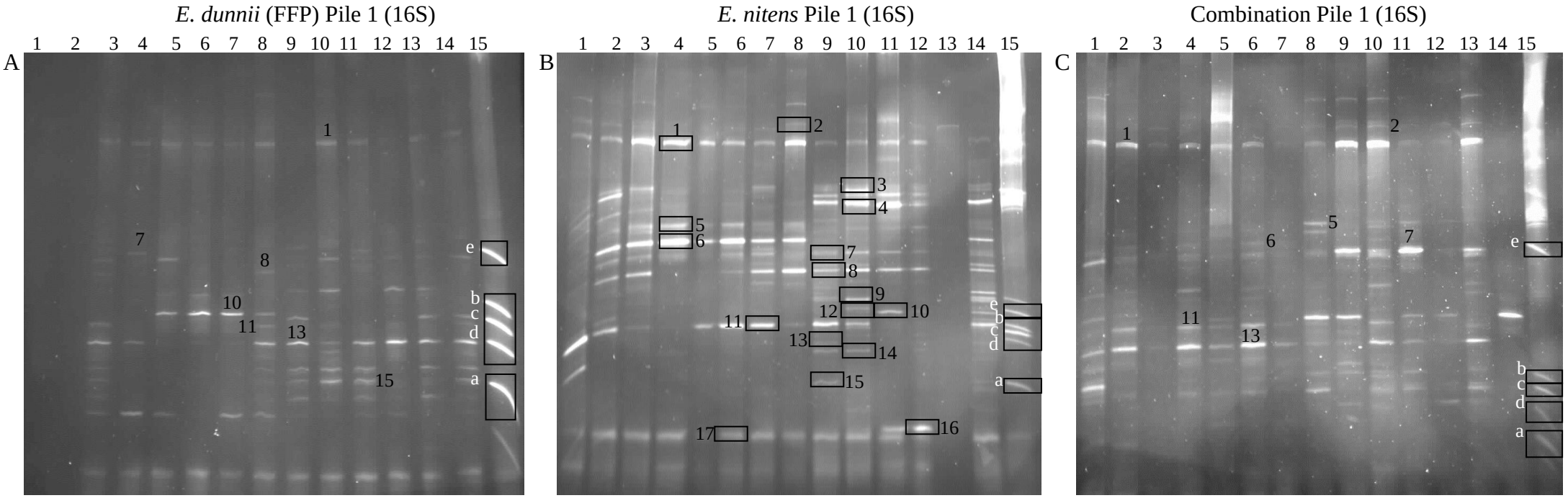

Figure 1: A-*E. dunnii* (FFP) Pile 1 (16S), B- *E. nitens* Pile 1 (16S), C- Combination Pile 1 (16S). Lanes 1: time zero, lane 2-4: 2 week sampling of winter simulation (section 1, 2 and 3), lane 5-7: 4 week sampling of winter simulation (section 1, 2 and 3), lane 8: time zero, lane 9-11: 2 week sampling of summer simulation (section1, 2 and 3), lane 12-14: 4 week sampling of summer simulation (section 1, 2 and 3), lane 15: known marker ([a]*Inquilinus limosus*, [b]*Klebsiella* sp, [c]*Pantoea* sp., [d]*Micrococcus luteus* and [e]*Pseudomonas aeruginosa*). Bands highlighted: 1-*Bacillus thuringiensis*, 2-Uncultured bacterium, 3-*Pseudomonas mohnii*, 4-*Shewanella* sp., 5-*Bacillus thuringiensis* Strain Se10, 6-*Bacillus* sp. R-43588, 7-*Erwinia* sp., 8-*Pantoea agglomerans*, 9-*Clavibacter michiganensis*, 10-M*ethylobacterium* sp., 11-*Gemella sanguinis*, 12-Anoxygenic photosynthetic bacterium, 13-Uncultured bacterium, 14-*Arthrobacter globiformis*, 15-*Modestobacter* sp., 16-Uncultured *Mycobacterium* sp., 17-*Bacillus* sp. NBSL38.

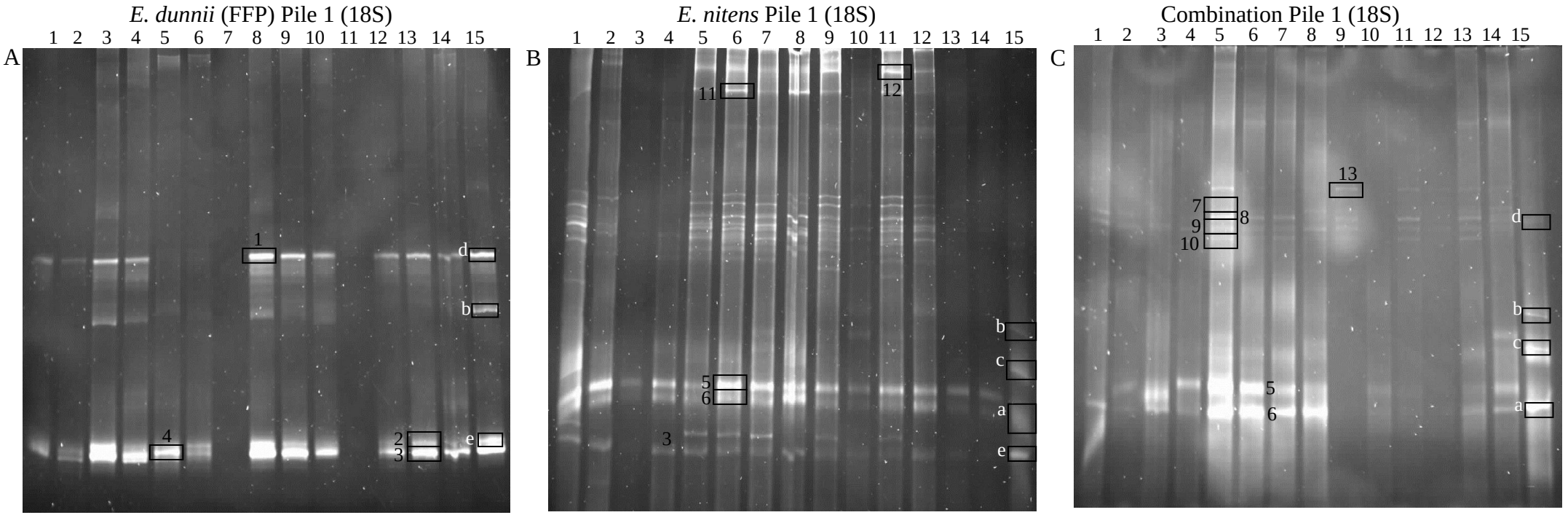

Figure 2: A-*E. dunnii* (FFP) Pile 1 (18S), B- *E. nitens* Pile 1 (18S), C- Combination Pile 1 (18S). Lanes 1: time zero, lane 2-4: 2 week sampling of winter simulation (section 1, 2 and 3), lane 5-7: 4 week sampling of winter simulation (section 1, 2 and 3), lane 8: time zero, lane 9-11: 2 week sampling of summer simulation (section1, 2 and 3), lane 12-14: 4 week sampling of summer simulation (section 1, 2 and 3), lane 15: known marker ([a]*Aspergillus fumigatus*, [b]*Paecilomyces formosus*, [c]*Phialophora alba*, [d]*Curvularia* sp. and [e]*Aspergillus fumigatus*). Bands highlighted: 1-Uncultured soil fungus, 2-Uncultured fungus FT09P23D12, 3-*Penicillium decumbens* s1821, 4-*Aspergillus nomius*, 5-*Lodderomyces elongisporus*, 6-*Penicillium decumbens*, 7-*Mytilinidion mytilinellum*, 8-*Normandia pulchella*, 9-*Symbiotaphrina buchneri*, 10-*Ascomycete* sp., 11-Uncultured *Paecilomyces* sp., 12- Uncultured eukaryote, 13- *Dacrymyces variisporus*.

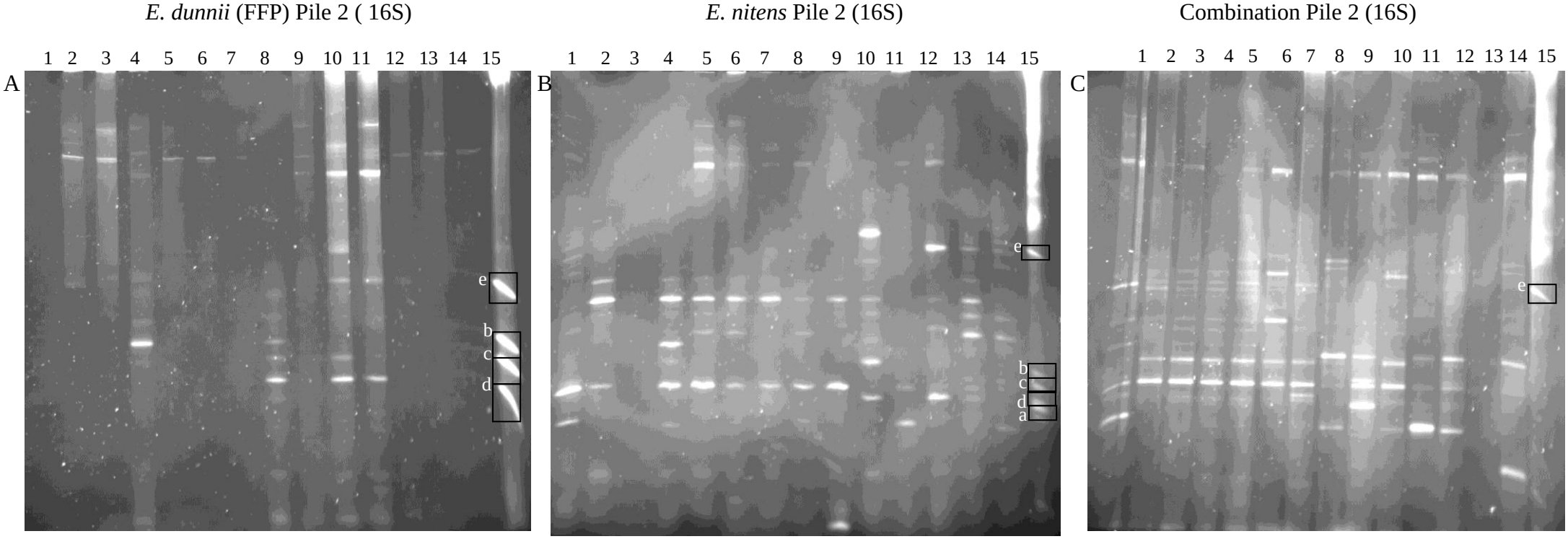

Figure 3: A-*E. dunnii* (FFP) Pile 2 (16S), B- *E. nitens* Pile 2 (16S), C- Combination Pile 2 (16S). Lanes 1: time zero, lane 2-4: 2 week sampling of winter simulation (section 1, 2 and 3), lane 5-7: 4 week sampling of winter simulation (section 1, 2 and 3), lane 8: time zero, lane 9-11: 2 week sampling of summer simulation (section1, 2 and 3), lane 12-14: 4 week sampling of summer simulation (section 1, 2 and 3), lane 15: known marker ([a]*Inquilinus limosus*, [b]*Klebsiella* sp, [c]*Pantoea* sp., [d]*Micrococcus luteus* and [e]*Pseudomonas aeruginosa*).

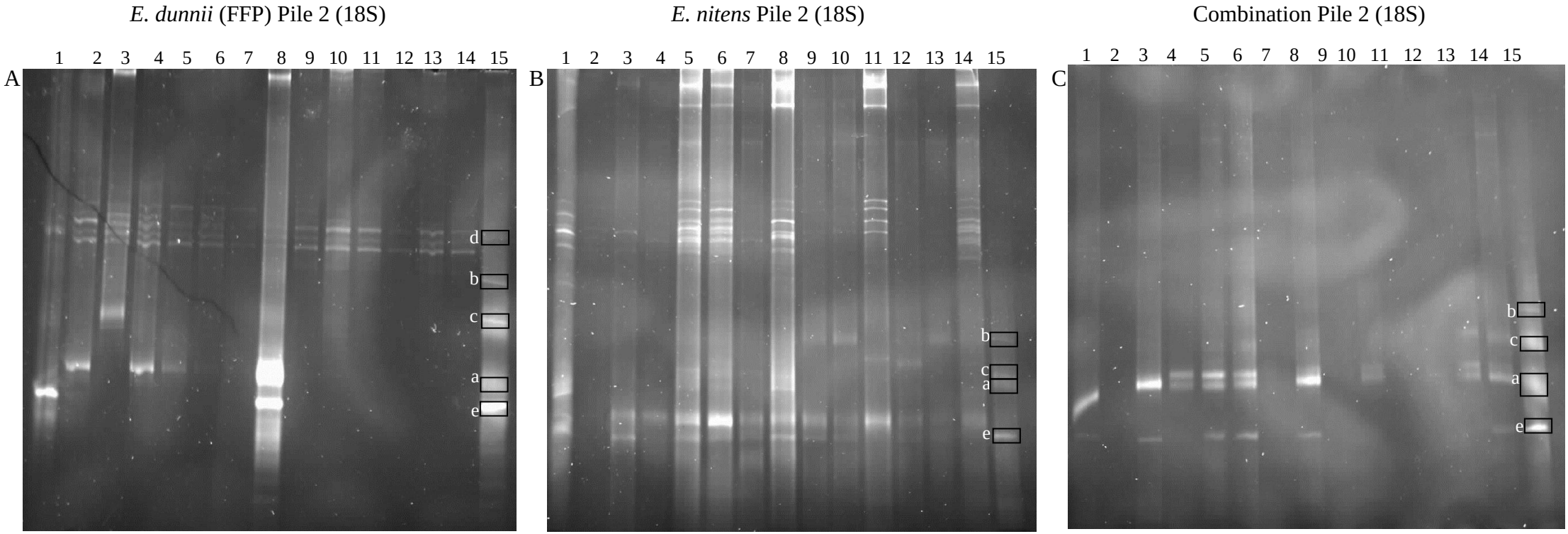

Figure 4: A-*E. dunnii* (FFP) Pile 2 (18S), B- *E. nitens* Pile 2 (18S), C- Combination Pile 2 (18S). Lanes 1: time zero, lane 2-4: 2 week sampling of winter simulation (section 1, 2 and 3), lane 5-7: 4 week sampling of winter simulation (section 1, 2 and 3), lane 8: time zero, lane 9-11: 2 week sampling of summer simulation (section1, 2 and 3), lane 12-14: 4 week sampling of summer simulation (section 1, 2 and 3), lane 15: known marker ([a]*Aspergillus fumigatus*, [b]*Paecilomyces formosus*, [c]*Phialophora alba*, [d]*Curvularia* sp. and [e]*Aspergillus fumigatus*)

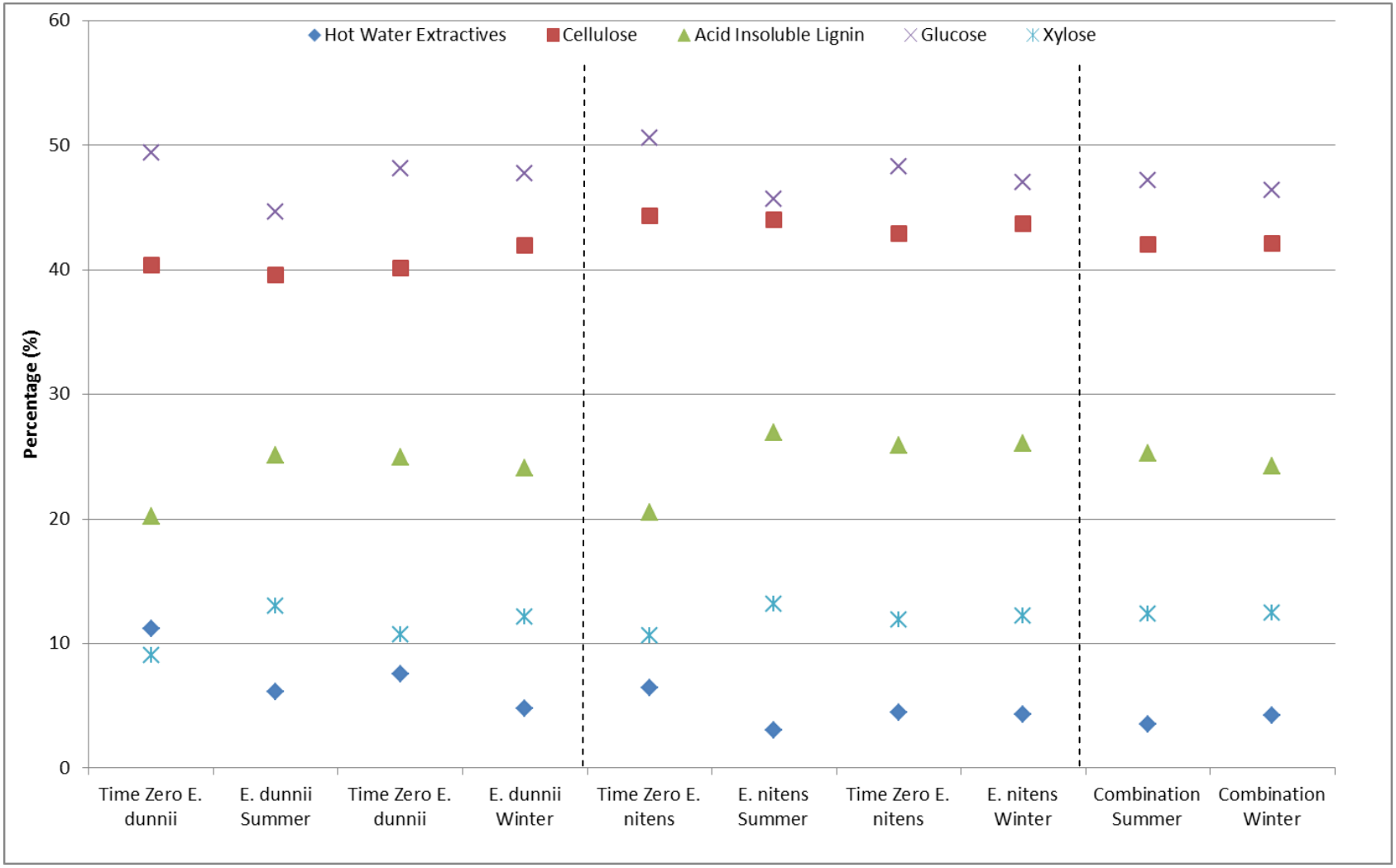

Figure 5: Chemical analysis of wood chips of *E. nitens*, *E. dunnii* and the Combination sampled from chip piles after summer and winter simulations.

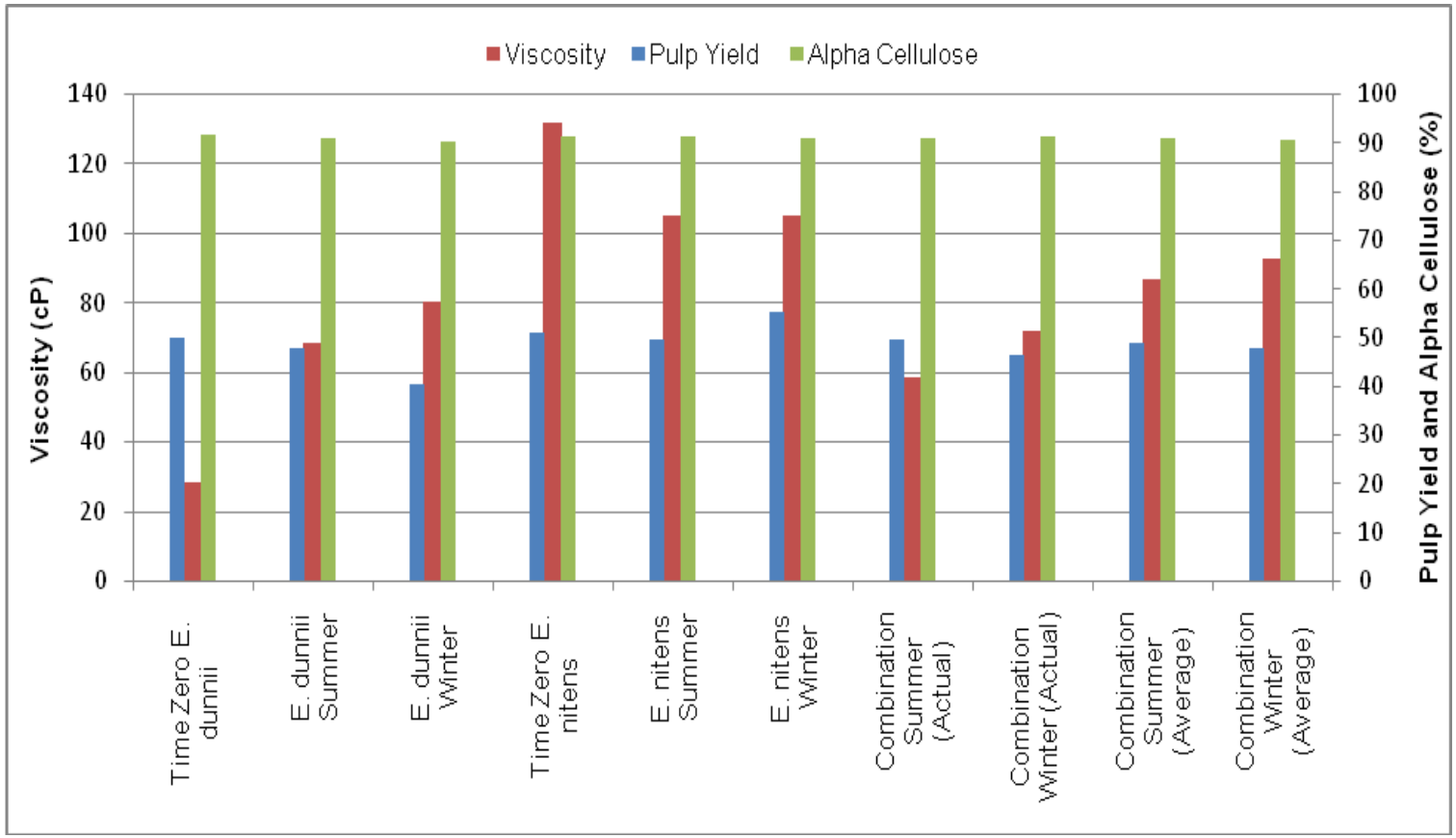

Figure 6: Viscosity, pulp yield and alpha cellulose content of acid bisulphite raw pulp produced from *E. dunnii*, *E. nitens* and the Combination wood chips after summer and winter simulations

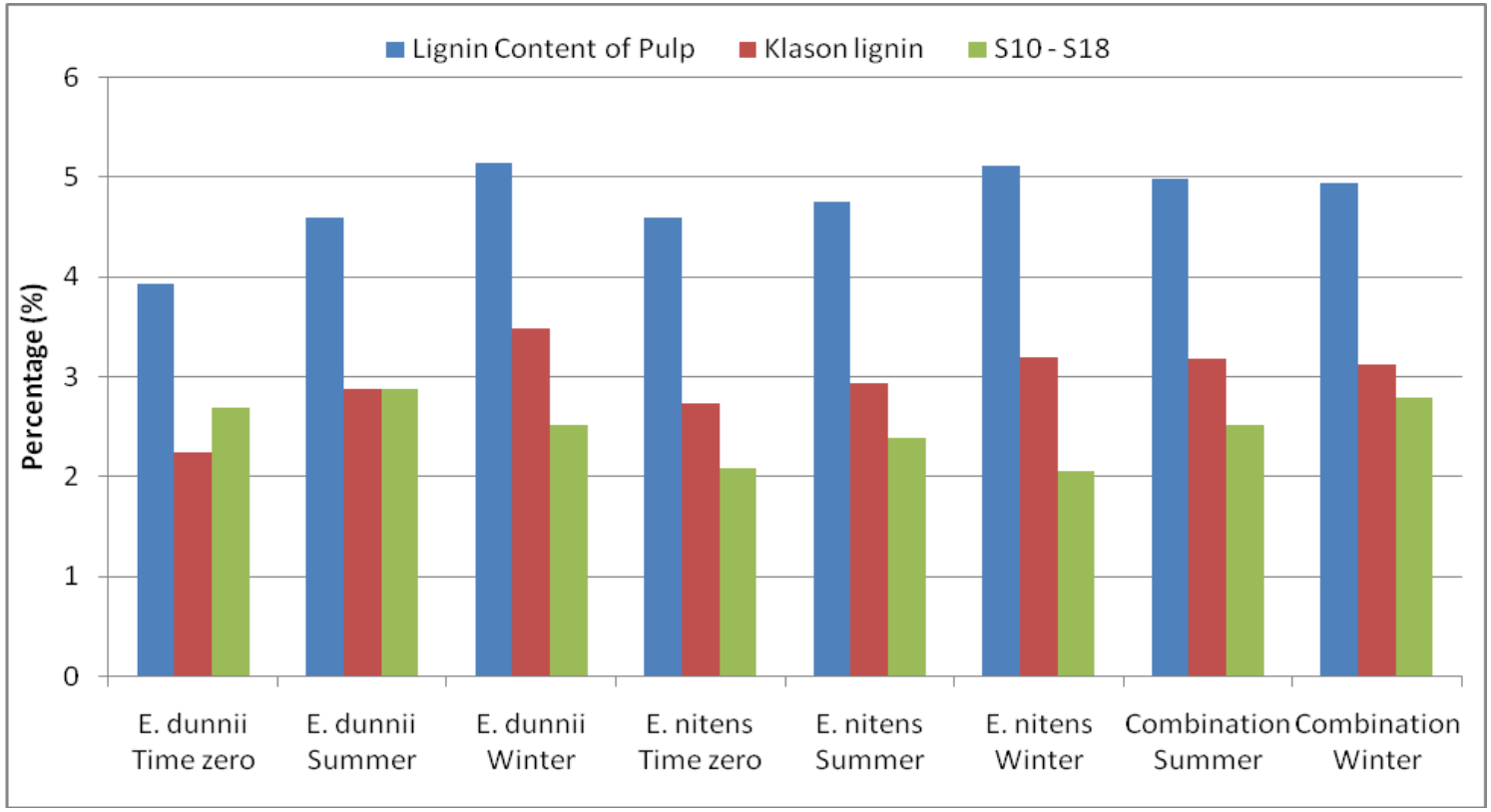

Figure 7: Total lignin, klason lignin and S10-S18 of acid bisulphite raw pulp produced from *E. dunnii*, *E. nitens* and the Combination wood chips after summer and winter simulations

5.1 The research in perspective

Since the inception of outdoor storage of wood chip piles, numerous external and internal variables have conspired to cause degradation and spontaneous combustion of these piles. Factors such as indigenous microflora, physical and chemical characteristics of the wood, cultivation environment, storage area and conditions contribute to the final quantity and quality of pulp generated. Controlling these factors has always been a challenge to foresters and mill personnel. The enormous potential for biofuel production from wood material has motivated researchers to investigate various methods of improving storage and biodegradation of outdoor wood chip piles to enhance biofuel generation. However, the requirements for biofuel production and biopulping vary significantly. Degradation of the major plant cell wall components (cellulose, hemicellulose and lignin) is necessary for the production of biofuels, whereas in biopulping, the reduction or removal of hemicellulose and lignin with intact cellulose fibres are essential. In the production of dissolving pulp the presence of residual hemicellulose and lignin is particularly undesirable as it becomes problematic in downstream processing. Indigenous microflora isolated from wood material has shown enormous potential as agents in biopulping or biobleaching. There are several discrepancies in this area of research, pertaining to the contributing effects of indigenous microflora to pulp properties as well as seasonal variations of microflora and their effects, in combination with changes in composition of the wood, on the pulp. Previous studies have focussed on either the isolation of particular microorganisms or bacteria or fungi from wood, and in several cases, applying the traditional culturing technique for identification of microflora. To address these pitfalls, this study reports on the seasonal variations of microflora in a commercial woodchip pile and their potential relationships with the chemical composition of the woodchips. Chemical characteristics and indigenous microflora of individual *Eucalyptus* species and a combination of these species were also investigated. Pulping data from this aspect of the study enabled correlations between, wood chip qualities, chemical and physical properties of the wood species and their microflora before and after exposure to simulated weather conditions.

The first phase of this study involved optimization of the PCR-DGGE method to obtain a standardized method for the evaluation of indigenous microflora present in hardwood chips intended for pulping. Factors influencing this technique include primer selection and gel gradients. In order to address these parameters various primers and gradients were tested. Optimal primer sets were 338f-GC/518r and 18S-NS26f/518r-GC for bacteria and fungi,

respectively. Optimum gradients for the separation of amplicons on DGGE gels were 30/60% and 25/50% for bacteria and fungi, respectively. Limitations encountered in the application of this technique were reduced visibility and close proximity of bands resulting in unidentified species (Maarit-Niemi et al. 2001; Xue et al. 2008). These problems may be countered by overloading the gel and varying the gradients to ensure optimal separation of those regions. Initially manual methods of DNA extraction from the wood chips were employed, however, it was believed that plant phenols were inhibiting the PCR reaction which ultimately led to the implementation of a commercial DNA extraction kit with purification columns. The application DGGE facilitated the identification of a greater number of isolates compared to the basic culture-dependent technique tested, thus branding it a highly favourable technique in the analysis of microbial communities in wood chip piles.

The assessment in summer and winter of a cross section of commercial wood chip piles in this study provided insight into the microbial diversity present in different areas of the pile in different seasons. Bacterial species appeared to dominate all areas of the wood chip piles both in summer and winter. Bacteria are initial colonizers of woods chips and compost (Clausen 1996; Fuller, 1985). The commercial pulping mill from which samples were collected requires approximately 1 week for the construction of a 20 m wood chip pile and 2 weeks to reclaim it for pulping, therefore the prevalence of bacteria is not unexpected as the chips remain stagnant for a short period of time. Studies with extended storage times revealed a wider variety of bacteria and fungi (Brand et al. 2007, 2010; Jirjis et al. 2008; Raberg et al. 2009). Minimal variations were observed for the summer and winter samples possibly due to smaller pile heights and reduced storage time (< 1 month). In addition, no problems with final pulp quality or yield were reported during this sampling period. The warmer ambient temperatures (25ºC) and humidity (64%) in summer may have contributed to the greater variety and wealth bacterial and fungal species observed. The identification of unusual species in the wood chip piles such as *I. limosus* and *L. palmae* again highlights the advantages of applying DGGE for microbial community analyses compared to traditional culturing techniques. Common bacterial species such as *Streptococcus* sp., *Bacillus* sp. and *Klebsiella* spp. identified here have been reported in other studies (Clausen 1996; Greaves 1971). The ability of most of these common species to produce cellulases (Clausen 1996; Maki et al. 2009) is of concern, particularly in the dissolving pulp industry where the preservation of cellulose fibres is imperative for the generation of high quality pulp (Christov

et al. 1998). Lower cellulose content and higher percentage glucose in summer may be correlated with greater microbial activity as glucose levels increased indicating the degradation of cellulose. Removal of lignin is important in the dissolving pulping industry as it ensures purity of the alpha cellulose fibres thereby increasing quality of the pulp and facilitates downstream bleaching processing (Oluwadare and Asagbara 2008). *P. rodasii* could be the potential cause of lower acid insoluble lignin content in summer. Endophytic fungi are known to dominate healthy tissue of wood material and are proficient producers of cellulases, therefore they are instigators for wood decay (Choi et al. 2005). Further investigation of these endophytic fungi could improve current methods of biopulping. This phase of the study it was shown that there was seasonal variation of microflora within the chip pile. Significant correlations with wood chemistry, season and location in pile were observed, some of which were due to variations in microflora. Limitations encountered in this phase included the presence of undisclosed *Eucalyptus* species in the wood chip pile, as the inherent variations of each wood species adds complexity to the study, also the initial sampling strategy could not be performed due to restrictions in the woodyard.

In the final phase, wood chip piles exposed to simulated summer and winter conditions provided a valuable understanding of the contributions of the individual *Eucalyptus* species in terms of chemistry, microflora and pulp properties. Chemical composition of the wood is known to have an influence on the pulp yield, bleaching, and consumption of cooking liquor (Sappi, 2012). Differences in pulp quality were observed. due to the varying chemistry and indigenous microflora of each wood species. The species of wood and the age of the wood chips were reported to have an effect on decomposition rates (Blanchette and Shaw, 1978). Fuller (1985) proposed that mixing of wood species with different deterioration rates would reduce negative effects on pulp quality. This study provides proof of this theory by demonstrating an overall increase in the pulp yields, especially during the summer simulation. Positive influences of combining the two wood species were observed in winter with lower lignin and higher α-cellulose levels and in summer with higher yield and lower degraded cellulose. The positive effect was also evident in the significantly lower hot water extractive levels in the combination piles compared to the individual wood species. High hot water extractives levels relates to the concentration of lipophilic compounds in the pulp that results in the formation of pitch deposits in the pulp and on the machinery (Gutierrez et al., 1998). Minimal effects on the chemical composition of *E. dunnii* fuel wood chips was observed during storage (for up to four months) compared to pine chips (Brand et al., 2011), although moisture content decreased more rapidly.

Similarly, although storage had been shorter, minimal changes in wood chemistry were observed for *E. dunnii* and *E. nitens*.

Microbial populations in commercial wood chip piles are certainly involved in wood decay, however, the extent of their involvement and their contributing effects on final pulp quality and yield has yet to be elucidated. This study established microbial communities present in industrial scale hardwood chip piles intended for dissolving pulp production as well as individual *Eucalyptus* species and the succession during storage under winter and summer conditions. The understanding gained on the microbiological effects on commercial wood chip piles will be valuable in preventing major losses and exploitation of the positive influences of indigenous microflora which may assist in reducing chemical requirements. This could be a step forward in promoting environmental awareness within the industry and developing a proactive approach toward utilizing biological resources.

5.2 Potential for future development of the study

This is a primary study on the microbiological aspect of wood chip pile management motivated by a commercial pulping mill. Although results from this study provide novel information on occurrences within a wood chip pile, further insight into the effects of microflora on the wood chips and establishing trends between wood species, site quality, microflora, and chemical properties of wood chips would significantly benefit the pulping industry. Additional research into exposing microorganisms and their enzymes and their role in wood decay would be greatly beneficial to wood chip management. Additional studies should focus on evaluating the potential for biopulping by the microorganisms isolated from wood chip piles or their lignocelluloytic enzymes. The reduced hot water extractive level observed in this study provides an excellent opportunity to prospect for lipases and xylanases from indigenous microflora in the areas of interest. Isolation, purification and application of these enzymes may be the enhancement required to commercialize biopulping.

References

Blanchette, R. A. and C. G. Shaw. 1978. Associations among bacteria. Yeasts, and basidiomycetes during wood decay. *Phytopathology* **68:**631 – 637

Brand, M. A., G. I. B. de Muñiz, W. F. Quirino, and J. O. Brito. 2011. Storage as a tool to improve wood fuel quality. *Biomass and Bioenergy* **35:**2581 – 2588

Christov, L. P., M. Akhtar, and B. A. Prior. 1998. The potential of biosulfite pulping in dissolving pulp production. *Enzyme and Microbial Technology* **23:**70 – 74

Clausen, C. A. 1996. Bacterial associations with decaying wood: a review. *International Biodeterioration & Biodegradation* **37:**101 – 107

Fuller, W. S. 1985. Chip pile storage – a review of practices to avoid deterioration and economic losses. *Tappi Journal* **68:**48 – 52

Greaves, H. 1971. The Bacterial Factor in Wood Decay. *Wood Science and Technology* **5:**6 – 16

Gutiérrez, A., J. C. del Rio, and F. J. González-Vila. 1998. Variation in the composition of wood extractives from *Eucalyptus globulus* during seasoning. *Journal of Wood Chemistry and Technology* **184:**439 – 446

Jirjis, R., L. Pari, F. Sissot. 2008. Storage of poplar wood chips in Northern Italy. *World Bioenergy* **8:**85 – 89

Maarit-Niemi, R., I. Heiskanen, K. Wallenius, K. Lindstrom. 2001. Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. Journal of Microbiological Methods **45:**155 – 165

Maki, M., K. T. Leung, and W. Qin. 2009. The prospects of cellulose-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences* **5:**500 – 516

Oluwadare, A. O. and E. O. Asagbara. 2008. Biodegradation of *Sterculia setigera* (Sterculiaceae) chips and its effects on wood basic chemical composition. *International Journal of Botany* **4:**461 – 465

Råberg, U., N. Terziev, C. J. Land. 2009. Early soft rot colonization of Scots sapwood pine in above-ground exposure. *International Biodeterioration & Biodegradation* **63:**236-240

Sappi, Frequently asked questions about: Eucalyptus trees, July 2012. Available at: www.**sappi**.com/.../QU971_SAP_KS_FAQ_**Eucalyptus**_web-v4.pdf Accessed: 31.01.2013

Xue D, Yao H-Y, Ge D-Y, Huang C-Y. Soil microbial community structure in diverse land use systems: a comparative study using biolog, DGGE, and PLFA analyses. Pedosphere. 2008; 18:653-663.

APPENDIX A: CHEMISTRY OF WOOD

Table A1: Chemistry of wood chips collected from the commercial wood chip piles in summer and winter

	Date	Samples	Temp (°C)	Hot water Extractives %	Cellulose %	Acid Insoluble Lignin %	Arabinose %	Galactose %	Glucose %	Xylose %	Mannose %
WINTER	2 Aug '10	North_02.08	30	4.16	45.14	26.95	0.19	0.57	49.86	10.55	1.41
		North_02.08a		4.57	45.01	27.02	0.18	0.54	49.67	10.58	1.41
		North_02.08b		4.37	45.00	27.06	0.18	0.54	49.90	10.59	1.44
		South_02.08	28	3.64	44.35	26.16	0.15	0.52	49.88	10.58	1.51
		South_02.08a		3.18	44.31	26.27	0.16	0.57	50.43	10.57	1.45
		South_02.08b		3.06	44.56	25.83	0.15	0.60	50.29	10.48	1.51
		East_02.08	27	3.63	43.04	27.36	0.28	0.78	48.90	11.69	1.21
		East_02.08a		3.49	42.92	27.38	0.28	0.84	48.30	11.89	1.24
		East_02.08b		3.68	42.71	27.29	0.30	0.93	48.47	11.77	1.22
		West_02.08	22	3.57	44.12	27.21	0.11	0.63	50.62	10.38	1.51
		West_02.08a		3.85	43.98	27.12	0.11	0.71	50.51	10.33	1.49
		West_02.08b		3.71	43.94	27.27	0.12	0.69	50.74	10.35	1.51
		Middle_02.08	50	4.64	43.90	27.00	0.21	0.59	49.15	10.62	1.43
		Middle_02.08a		4.78	43.62	27.11	0.21	0.65	49.05	10.62	1.38
		Middle_02.08b		5.07	43.87	26.76	0.22	0.62	48.98	10.69	1.44
	3 Aug '10	North_03.08	35	4.54	44.72	26.98	0.20	0.57	50.22	10.48	1.42
		North_03.08a		4.56	44.76	27.19	0.20	0.61	50.32	10.50	1.38
		North_03.08b		4.15	44.47	27.09	0.20	0.64	50.46	10.41	1.42
		East_03.08	45	5.02	43.52	28.00	0.28	0.78	48.38	11.06	1.32
		East_03.08a		5.05	43.56	27.96	0.28	0.81	48.48	10.99	1.32
		East_03.08b		5.11	43.20	28.04	0.29	0.81	48.56	10.91	1.25
		South_03.08	40	3.39	45.22	25.90	0.12	0.62	50.92	9.94	1.55

South_03.08a		3.24	45.29	25.99	0.13	0.64	51.24	9.91	1.49
South_03.08b		2.71	44.89	26.10	0.13	0.65	51.64	9.91	1.49
Middle_03.08	42	4.81	44.87	27.22	0.19	0.63	50.72	9.93	1.31
Middle_03.08a		4.96	44.88	27.12	0.19	0.59	50.51	10.01	1.32
Middle_03.08b		4.82	44.83	27.08	0.20	0.63	50.40	9.96	1.32
West_03.08	33	5.01	43.86	27.94	0.20	0.51	49.17	10.38	1.39
West_03.08a		5.35	44.10	27.67	0.20	0.53	49.24	10.29	1.42
West_03.08b		5.28	43.94	27.74	0.21	0.52	49.25	10.33	1.43
Middle_03.08	27	3.20	44.41	26.25	0.13	0.58	49.95	10.80	1.59
Middle_03.08a		3.07	44.35	26.37	0.14	0.61	50.20	10.75	1.59
Middle_03.08b		2.87	44.25	26.76	0.13	0.59	50.23	10.73	1.54
North_03.08	48	3.79	43.80	26.26	0.18	0.56	49.13	11.12	1.52
North_03.08a		3.50	43.46	26.38	0.18	0.61	49.18	11.24	1.52
North_03.08b		3.40	43.67	26.26	0.17	0.58	49.47	11.15	1.55
West_03.08	34	4.77	43.28	26.90	0.24	0.80	48.89	10.47	1.32
West_03.08a		4.91	43.22	27.01	0.24	0.74	48.88	10.54	1.32
West_03.08b		4.77	43.27	26.84	0.25	0.83	48.74	10.55	1.33
South_03.08	34	4.44	43.75	26.36	0.21	0.59	48.68	11.13	1.39
South_03.08a		4.56	43.68	26.32	0.22	0.64	48.91	11.06	1.41
South_03.08b		4.49	43.77	26.32	0.21	0.63	48.70	11.07	1.45
East_03.08	27	3.91	43.56	26.27	0.15	0.59	49.34	11.09	1.53
East_03.08a		4.01	43.50	26.36	0.15	0.58	49.39	11.12	1.50
East_03.08b		4.23	43.42	26.35	0.17	0.63	49.38	11.07	1.48
Middle_03.08	25	4.37	43.91	26.07	0.18	0.56	48.21	11.27	1.56
Middle_03.08a		4.45	43.89	26.00	0.19	0.59	48.45	11.17	1.54
Middle_03.08b		4.20	43.66	26.05	0.19	0.61	48.53	11.21	1.53
North_03.08	34	3.58	44.74	26.60	0.18	0.64	49.99	10.45	1.48
North_03.08a		3.49	44.61	26.63	0.18	0.67	50.40	10.39	1.48

		North_03.08b		3.23	44.94	26.27	0.16	0.68	50.40	10.29	1.52
		West_03.08	28	3.22	44.80	26.34	0.13	0.64	50.17	10.59	1.57
		West_03.08a		2.97	44.95	26.29	0.11	0.57	50.19	10.59	1.58
		West_03.08b		2.74	44.89	26.20	0.10	0.58	50.51	10.56	1.60
		South_03.08	28	4.69	43.52	26.70	0.21	0.66	48.26	10.93	1.46
		South_03.08a		4.48	43.55	26.69	0.22	0.67	48.49	10.88	1.45
		South_03.08b		4.53	43.32	26.66	0.21	0.65	48.35	10.91	1.48
		East_03.08	28	2.69	43.88	26.72	0.12	0.57	50.09	11.00	1.59
		East_03.08a		2.29	44.08	26.69	0.11	0.59	50.12	11.01	1.57
		East_03.08b		2.53	44.27	26.50	0.10	0.54	50.35	10.83	1.62
	4 Aug '10	South_04.08	41	2.54	44.72	24.12	0.21	0.20	51.75	12.32	1.60
		South_04.08a		2.52	44.48	24.06	0.20	0.20	51.47	12.28	1.57
		South_04.08b		2.52	44.70	23.80	0.20	0.28	51.93	12.18	1.60
		Middle_04.08	32	4.69	43.81	27.17	0.18	0.69	48.71	11.42	1.37
		Middle_04.08a		4.71	43.76	27.08	0.17	0.70	49.31	11.37	1.36
		Middle_04.08b		4.35	43.72	26.93	0.16	0.72	49.18	11.30	1.41
		North_04.08	29	4.77	42.89	25.52	0.21	0.49	48.07	12.26	1.49
		North_04.08a		4.71	43.10	25.23	0.22	0.54	48.24	12.31	1.49
		North_04.08b		4.41	43.06	25.33	0.22	0.54	48.10	12.36	1.54
		West_04.08	30	2.95	44.33	24.76	0.14	0.48	50.47	11.59	1.66
		West_04.08a		3.06	44.32	24.74	0.13	0.46	50.19	11.62	1.67
		West_04.08b		3.01	44.47	24.45	0.13	0.51	50.65	11.48	1.67
		East_04.08	30	5.06	42.86	25.64	0.24	0.51	48.51	11.89	1.43
		East_04.08a		4.63	43.08	25.44	0.24	0.59	48.79	11.90	1.46
		East_04.08b		5.00	42.58	25.74	0.23	0.51	48.59	11.83	1.42
		North_04.08	30	4.03	44.48	27.17	0.20	0.54	50.44	10.40	1.33
		North_04.08a		3.92	44.58	27.11	0.20	0.57	50.59	10.36	1.32
		North_04.08b		3.81	44.41	27.01	0.20	0.56	50.41	10.40	1.37

		South_04.08	31	4.97	44.75	26.38	0.18	0.60	50.81	10.03	1.33
		South_04.08a		4.88	44.73	26.53	0.19	0.59	51.17	10.11	1.30
		South_04.08b		5.09	44.69	26.49	0.17	0.55	50.99	9.87	1.33
		Middle_04.08	31	3.55	43.64	26.70	0.20	0.57	50.36	10.66	1.42
		Middle_04.08a		3.83	44.00	26.63	0.20	0.53	50.06	10.74	1.42
		Middle_04.08b		3.65	43.61	26.55	0.21	0.64	50.31	10.64	1.41
		East_04.08	28	4.01	44.78	27.52	0.20	0.60	50.08	10.46	1.32
		East_04.08a		4.08	44.92	27.42	0.19	0.59	50.36	10.28	1.28
		East_04.08b		4.41	44.53	27.51	0.21	0.63	50.46	10.27	1.27
		West_04.08	28	3.63	44.95	26.96	0.22	0.81	50.36	10.82	1.16
		West_04.08a		3.29	45.18	26.89	0.20	0.79	50.44	10.80	1.20
		West_04.08b		3.31	45.17	26.85	0.20	0.77	51.07	10.71	1.19
SUMMER	1 Feb '11	North 01/02/11	44	5.54	44.47	23.87	0.19	0.83	51.08	9.53	1.62
		North 01/02/11a		5.74	44.62	24.18	0.18	0.78	51.12	9.54	1.59
		North 01/02/11b		5.72	44.12	24.19	0.19	0.81	51.13	9.58	1.64
		South 01/02/11	33	5.21	43.85	23.45	0.26	0.90	49.98	10.59	1.61
		South 01/02/11a		4.83	43.84	23.50	0.25	0.92	50.10	10.60	1.58
		South 01/02/11b		4.86	43.57	23.28	0.26	0.99	50.34	10.52	1.61
		East 01/02/11	39	5.32	43.51	25.27	0.22	0.87	49.47	10.13	1.65
		East 01/02/11a		5.46	43.47	25.33	0.23	0.90	49.74	10.12	1.63
		East 01/02/11b		5.21	43.50	25.18	0.22	0.88	49.50	10.19	1.67
		West 01/02/11	33	8.44	43.48	19.97	0.35	1.31	50.44	8.47	1.95
		West 01/02/11a		8.00	43.54	20.34	0.33	1.28	50.21	8.70	1.93
		West 01/02/11b		8.25	43.37	20.74	0.32	1.21	50.14	8.74	1.90
		Middle 01/02/11	32	5.71	44.01	24.57	0.22	1.05	50.73	9.83	1.47
		Middle 01/02/11a		5.88	43.95	24.65	0.21	0.97	50.42	9.93	1.48
		Middle 01/02/11b		5.61	43.63	24.59	0.22	1.08	50.62	9.87	1.49
		North 01/02/11	32	6.66	43.27	24.01	0.22	0.92	49.30	9.69	1.58

		North 01/02/11a		6.56	43.22	24.10	0.23	0.95	49.47	9.67	1.56
		North 01/02/11b		6.40	42.94	24.07	0.23	0.97	49.48	9.72	1.59
		South 01/02/11	39	6.47	42.77	23.19	0.30	1.04	49.60	9.64	1.63
		South 01/02/11a		6.58	42.70	23.31	0.29	0.97	49.57	9.68	1.61
		South 01/02/11b		6.22	42.48	23.14	0.30	1.07	49.50	9.70	1.67
		East 01/02/11	31	6.98	44.40	23.21	0.18	0.80	49.99	9.65	1.71
		East 01/02/11a		6.66	44.18	22.95	0.21	0.83	50.14	9.66	1.69
		East 01/02/11b		7.09	43.94	22.91	0.21	0.87	50.18	9.60	1.72
		West 01/02/11	35	5.94	43.91	24.70	0.20	0.94	50.18	9.62	1.58
		West 01/02/11a		6.01	43.69	24.77	0.21	0.88	50.13	9.73	1.59
		West 01/02/11b		5.79	43.62	24.63	0.21	0.96	50.30	9.66	1.61
		Middle 01/02/11	31	5.77	42.59	25.65	0.27	1.12	48.40	11.00	1.34
		Middle 01/02/11a		6.09	42.94	25.62	0.27	1.08	48.22	10.97	1.37
		Middle 01/02/11b		5.33	42.29	25.76	0.27	1.19	48.42	11.05	1.38
	2 Feb '11	North 02/02/11	34	4.09	43.99	25.95	0.26	1.12	49.32	10.97	1.28
		North 02/02/11a		4.24	43.83	25.86	0.27	1.17	49.01	11.04	1.28
		North 02/02/11b		3.29	43.69	25.88	0.28	1.24	49.29	11.09	1.25
		South 02/02/11	31	5.95	43.97	23.33	0.24	0.90	51.47	9.20	1.69
		South 02/02/11a		5.85	44.00	23.45	0.24	0.89	51.40	9.29	1.67
		South 02/02/11b		5.81	43.82	23.63	0.24	0.89	51.33	9.34	1.65
		East 02/02/11	31	4.36	43.79	26.16	0.25	1.04	49.23	10.82	1.29
		East 02/02/11a		4.25	43.61	26.24	0.25	1.06	49.23	10.86	1.34
		East 02/02/11b		4.16	43.44	26.26	0.25	1.05	49.23	10.84	1.35
		West 02/02/11	32	5.44	44.41	23.73	0.21	0.96	51.82	8.94	1.60
		West 02/02/11a		5.34	44.21	23.57	0.22	0.97	51.91	8.95	1.69
		West 02/02/11b		5.52	44.05	23.21	0.23	1.07	52.26	8.71	1.67
		Middle 02/02/11	30	6.42	43.56	22.05	0.31	1.11	50.53	9.20	1.73
		Middle 02/02/11a		6.33	43.58	22.48	0.29	1.06	50.72	9.21	1.70
		Middle 02/02/11b		5.92	43.48	22.82	0.27	1.04	50.44	9.45	1.65

		North 02/02/11	34	6.80	44.81	23.01	0.28	1.23	51.33	8.15	1.72
		North 02/02/11a		6.90	44.90	23.25	0.26	1.18	51.26	8.21	1.71
		North 02/02/11b		6.65	44.51	23.38	0.26	1.20	51.34	8.29	1.70
		South 02/02/11	41	6.02	44.02	23.65	0.23	0.96	50.60	9.61	1.63
		South 02/02/11a		6.41	44.11	23.37	0.23	0.97	50.70	9.50	1.64
		South 02/02/11b		6.07	43.99	23.34	0.24	0.98	50.66	9.55	1.65
		East 02/02/11	33	5.97	43.61	24.78	0.20	0.80	50.79	9.39	1.68
		East 02/02/11a		6.12	43.60	24.47	0.21	0.80	50.73	9.38	1.69
		East 02/02/11b		6.12	43.58	24.70	0.21	0.77	50.80	9.29	1.67
		West 02/02/11	33	6.04	43.63	24.45	0.22	0.71	50.48	9.94	1.68
		West 02/02/11a		6.09	43.99	23.90	0.21	0.74	51.13	9.54	1.74
		West 02/02/11b		6.23	43.70	23.33	0.24	0.82	50.81	9.61	1.81
		Middle 02/02/11	47	4.20	43.98	25.69	0.25	1.14	50.09	10.58	1.31
		Middle 02/02/11a		4.18	44.13	25.58	0.26	1.13	49.89	10.66	1.30
		Middle 02/02/11b		4.01	43.59	25.70	0.26	1.21	50.14	10.58	1.31
	3 Feb '11	North 03/02/11	34	4.46	43.57	25.95	0.25	0.88	48.86	11.18	1.38
		North 03/02/11a		4.47	43.87	25.78	0.24	0.89	49.08	11.08	1.39
		North 03/02/11b		4.33	43.41	26.02	0.24	0.90	48.89	11.13	1.39
		South 03/02/11	35	5.36	43.52	24.92	0.22	0.79	50.14	9.97	1.61
		South 03/02/11a		5.17	43.96	25.13	0.21	0.78	49.97	10.13	1.56
		South 03/02/11b		5.31	43.71	24.86	0.22	0.80	50.36	9.89	1.63
		East 03/02/11	32	5.61	42.88	26.34	0.20	0.83	48.86	10.17	1.60
		East 03/02/11a		5.38	43.10	26.19	0.21	0.88	49.01	10.17	1.63
		East 03/02/11b		5.44	43.06	26.06	0.20	0.87	48.93	10.20	1.66
		West 03/02/11	31	5.97	43.88	24.57	0.21	0.81	50.72	9.47	1.65
		West 03/02/11a		5.69	43.69	24.77	0.21	0.74	50.97	9.53	1.65
		West 03/02/11b		5.58	43.72	24.84	0.19	0.81	50.77	9.49	1.66
		Middle 03/02/11	34	5.12	43.97	25.89	0.20	0.78	50.49	10.19	1.53
		Middle 03/02/11a		4.76	44.04	25.98	0.20	0.84	50.38	10.17	1.53

		West 03/02/11b		4.71	43.89	25.66	0.19	0.82	50.53	10.19	1.57
		North 03/02/11	34	5.25	44.14	24.64	0.22	0.78	50.74	10.28	1.60
		North 03/02/11a		5.03	44.41	24.77	0.21	0.85	50.74	10.26	1.61
		North 03/02/11b		4.81	44.29	24.76	0.21	0.83	50.86	10.26	1.60
		South 03/02/11	36	6.11	43.22	23.55	0.25	0.89	49.95	9.85	1.68
		South 03/02/11a		6.55	43.10	23.34	0.26	0.88	49.84	9.78	1.69
		South 03/02/11b		6.25	43.16	23.75	0.26	0.91	50.02	9.87	1.63
		East 03/02/11	34	5.34	43.55	25.50	0.20	0.82	49.95	10.10	1.55
		East 03/02/11a		5.45	43.33	25.63	0.21	0.78	49.67	10.20	1.51
		East 03/02/11b		5.27	43.26	25.52	0.20	0.82	49.70	10.15	1.57
		West 03/02/11	48	5.50	43.61	25.16	0.22	0.96	49.78	9.74	1.64
		West 03/02/11a		5.82	43.55	25.25	0.21	0.88	49.79	9.70	1.65
		West 03/02/11b		5.45	43.51	25.39	0.20	0.91	49.81	9.76	1.63
		Middle 03/02/11	32	5.61	43.92	24.40	0.22	0.73	50.57	9.92	1.65
		Middle 03/02/11a		5.84	44.10	24.33	0.22	0.77	50.32	9.95	1.72
		Middle 03/02/11b		5.83	43.90	24.18	0.22	0.83	50.64	9.80	1.70

Table A2: Chemistry of *E. dunnii* chips in the simulated and control piles

	Samples	Hot water Extractives %	Cellulose %	Acid Insoluble Lignin %	Arabinose %	Galactose %	Glucose %	Xylose %	Mannose %
Time 0	*E. dunnii*	7.59	40.33	24.96	0.31	1.14	48.04	10.69	1
	E. dunnii a	7.45	40.12	25.08	0.32	1.1	48.13	10.72	0.98
	E. dunnii b	7.56	39.92	24.91	0.32	1.21	48.11	10.64	1.02
Control Sappi (S1)	*E. dunnii*_section1 Sappi_02.09	7.28	39.37	24.32	0.36	1.81	46.53	12.04	0.75
	*E. dunnii*_section1 Sappi_02.09a	7.73	39.49	24.52	0.34	1.65	45.91	12.14	0.69
	*E. dunnii*_section1 Sappi_02.09b	6.87	38.09	24.8	0.37	1.97	46.49	12.08	0.77
	*E. dunnii*_section2 Sappi_02.09	7.23	39.11	25.13	0.33	1.59	48.42	10.71	0.94
	*E. dunnii*_section2 Sappi_02.09a	6.95	38.94	25.25	0.34	1.62	48.6	10.81	0.88
	*E. dunnii*_section2 Sappi_02.09b	7.1	38.43	24.89	0.33	1.64	48.48	10.61	1
	*E. dunnii*_section3 Sappi_02.09	6.92	38.97	24.58	0.36	1.78	45.79	12.54	0.73
	*E. dunnii*_section3 Sappi_02.09a	7.36	38.77	24.73	0.36	1.71	45.22	12.65	0.66
	*E. dunnii*_section3 Sappi_02.09b	7	38.84	24.44	0.37	1.82	46.19	12.42	0.75
Control Sappi (S2)	*E. dunnii*_control_section1 s2_Sappi 16.09.10	7.61	39.24	25.47	0.37	1.51	43.92	13.18	0.59
	*E. dunnii*_control_section1 s2_Sappi 16.09.10a	7.86	39.01	25.33	0.38	1.53	44.17	13.14	0.57
	*E. dunnii*_control_section1 s2_Sappi 16.09.10b	7.66	38.52	25.54	0.38	1.58	43.71	13.29	0.6
	*E. dunnii*_control_section2 s2_Sappi 16.09.10	7.46	38.82	24.41	0.43	1.73	44.93	12.75	0.77
	*E. dunnii*_control_section2 s2_Sappi 16.09.10a	7.7	38.81	24.67	0.44	1.73	44.52	12.84	0.75
	*E. dunnii*_control_section2 s2_Sappi 16.09.10b	7.57	38.51	24.55	0.43	1.76	44.59	12.76	0.77
	*E. dunnii*_control_section3 s2_Sappi 16.09.10	7.93	38.85	25.46	0.39	1.52	43.24	13.3	0.7
	*E. dunnii*_control_section3 s2_Sappi 16.09.10a	8.45	38.39	25.66	0.4	1.58	42.68	13.35	0.68
	*E. dunnii*_control_section3 s2_Sappi 16.09.10b	7.87	38.7	25.3	0.4	1.56	43.11	13.37	0.68
Control UKZN (S1)	*E. dunnii*_control_section1 s1_UKZN 14.09	5.77	39.43	24.16	0.39	1.93	45.88	12.67	0.76
	*E. dunnii*_control_section1 s1_UKZN 14.09a	5.74	39.12	24.36	0.39	1.92	45.85	12.63	0.77
	*E. dunnii*_control_section1 s1_UKZN 14.09b	5.7	39.15	24.24	0.39	1.96	45.96	12.56	0.78
	*E. dunnii*_control_section2 s1_UKZN 14.09	6.04	39.7	24.31	0.34	1.56	50.07	10.57	0.98

	*E. dunnii*_control_section2 s1_UKZN 14.09a	6.48	39.75	24.37	0.33	1.48	49.76	10.55	1
	*E. dunnii*_control_section2 s1_UKZN 14.09b	6.14	39.48	24.18	0.35	1.62	50.28	10.44	1.05
	*E. dunnii*_control_section3 s1_UKZN 14.09	7.84	39.76	24.26	0.35	1.4	48.28	10.88	0.93
	*E. dunnii*_control_section3 s1_UKZN 14.09a	7.96	39.74	24.34	0.38	1.48	48.08	10.94	0.89
	*E. dunnii*_control_section3 s1_UKZN 14.09b	7.53	38.7	24.08	0.39	1.7	48.34	10.74	1.01
Control UKZN (S2)	*E. dunnii*_control_section1_UKZN s2 28.09.10	9.82	36.05	23.92	0.49	1.49	45.45	10.16	0.82
	*E. dunnii*_control_section1_UKZN s2 28.09.10a	9.92	35.89	22.94	0.53	1.65	46.3	9.59	0.96
	*E. dunnii*_control_section1_UKZN s2 28.09.10b	9.92	35.65	23.02	0.52	1.59	45.88	9.73	0.96
	*E. dunnii*_control_section2_UKZN s2 28.09.10	7.68	38.77	23.31	0.33	1.27	48.49	9.74	1.15
	*E. dunnii*_control_section2_UKZN s2 28.09.10a	8.52	39.18	20.74	0.4	1.41	49.37	8.73	1.39
	*E. dunnii*_control_section2_UKZN s2 28.09.10b	7.37	38.73	22.27	0.36	1.42	49.11	9.37	1.24
	*E. dunnii*_control_section3_UKZN s2 28.09.10	7.87	37.85	23.56	0.37	1.27	47.96	10.1	1.05
	*E. dunnii*_control_section3_UKZN s2 28.09.10a	8.28	37.55	22.29	0.4	1.48	48.58	9.5	1.16
	*E. dunnii*_control_section3_UKZN s2 28.09.10b	7.89	37.47	23.33	0.37	1.33	48.04	9.98	1.06
Time 0	*E. dunnii*_time1_FFP_13.10.10	10.91	40.27	20.75	0.4	1.38	49.3	9.23	1.26
	*E. dunnii*_time1_FFP_13.10.10a	11.86	40.69	19.98	0.41	1.39	49.2	9.05	1.34
	*E. dunnii*_time1_FFP_13.10.10b	10.9	40.14	19.92	0.43	1.53	49.69	8.88	1.32
Winter (S1)	*E. dunnii*_winter_pile1_sect1	8.67	38.69	24.24	0.42	1.76	45.1	12.35	0.73
	*E. dunnii*_winter_pile1_sect1a	8.23	38.68	24.21	0.42	1.73	45.17	12.32	0.69
	*E. dunnii*_winter_pile1_sect1b	8.5	38.12	24.19	0.42	1.88	45.21	12.25	0.78
	*E. dunnii*_winter_pile1_sect2	8.3	39	24.42	0.39	1.65	48.15	10.52	0.91
	*E. dunnii*_winter_pile1_sect2a	7.89	39.19	24.6	0.37	1.58	48.41	10.61	0.87
	*E. dunnii*_winter_pile1_sect2b	7.77	38.69	24.17	0.37	1.72	49.02	10.31	0.99
	*E. dunnii*_winter_pile1_sect3	7.28	39.67	24.46	0.33	1.51	48.44	11.44	1.06
	*E. dunnii*_winter_pile1_sect3a	7.33	40.36	24.27	0.3	1.38	48.14	11.44	0.99
	*E. dunnii*_winter_pile1_sect3b	6.82	39.74	24.21	0.31	1.55	48.77	11.35	1.06
	*E. dunnii*_winter_pile2_sect1	6.26	38.5	24.02	0.38	2.06	46.71	12.01	0.89
	*E. dunnii*_winter_pile2_sect1a	7.29	40.05	24.21	0.35	1.66	45.25	12.37	0.7
	*E. dunnii*_winter_pile2_sect1b	6.22	38.61	24.14	0.36	1.97	46.44	12.08	0.88

	*E. dunnii*_winter_pile2_sect2	7.2	39.77	24.15	0.28	1.22	49.04	10.42	1.11
	*E. dunnii*_winter_pile2_sect2a	6.68	40.03	24.46	0.26	1.15	49.05	10.65	1.07
	*E. dunnii*_winter_pile2_sect2b	7.27	39.62	23.94	0.27	1.29	48.92	10.3	1.16
	*E. dunnii*_winter_pile2_sect3	6.46	40.51	23.54	0.31	1.57	47.25	12.34	0.95
	*E. dunnii*_winter_pile2_sect3a	6.24	40.51	23.69	0.29	1.43	46.97	12.55	0.95
	*E. dunnii*_winter_pile2_sect3b	5.82	40.36	23.38	0.3	1.59	47.29	12.39	0.96
	*E. dunnii*_winter_pile3_sect1	5.88	40.13	24.45	0.3	1.56	46.43	12.55	0.84
	*E. dunnii*_winter_pile3_sect1a	5.79	40.3	24.31	0.29	1.55	46.31	12.55	0.81
	*E. dunnii*_winter_pile3_sect1b	5.73	40.28	24.11	0.29	1.59	46.79	12.35	0.9
	*E. dunnii*_winter_pile3_sect2	5.96	39.95	24.01	0.28	1.52	47.25	12.07	0.94
	*E. dunnii*_winter_pile3_sect2a	6.45	40.53	23.79	0.28	1.43	46.91	12.15	0.89
	*E. dunnii*_winter_pile3_sect2b	5.66	38.6	23.66	0.32	1.89	47.71	11.86	1.02
	*E. dunnii*_winter_pile3_sect3	6.02	40.48	23.8	0.29	1.63	46.89	12.49	0.93
	*E. dunnii*_winter_pile3_sect3a	6.1	41.2	23.91	0.26	1.45	46.83	12.5	0.95
	*E. dunnii*_winter_pile3_sect3b	5.78	40.39	23.89	0.27	1.6	46.68	12.5	0.96
Winter (S2)	*E. dunnii*_winter_sim_pile1_sect1 s2	6.9	39.89	23.76	0.3	1.46	46.44	12.15	0.98
	*E. dunnii*_winter_sim_pile1_sect1 s2a	6.47	39.45	23.75	0.29	1.49	46.98	12.08	0.99
	*E. dunnii*_winter_sim_pile1_sect1 s2b	6.67	40.35	23.46	0.28	1.4	47.13	12	0.97
	*E. dunnii*_winter_sim_pile1_sect2 s2	6.52	40.57	23.4	0.25	1.15	49.96	10.98	1.17
	*E. dunnii*_winter_sim_pile1_sect2 s2a	6.54	40.3	23.32	0.24	1.22	50.18	10.86	1.26
	*E. dunnii*_winter_sim_pile1_sect2 s2b	6.63	40.35	23.34	0.25	1.18	50.11	10.91	1.22
	*E. dunnii*_winter_sim_pile1_sect3 s2	5.41	40.98	23.76	0.25	1.41	48.17	12.02	1.04
	*E. dunnii*_winter_sim_pile1_sect3 s2a	5.53	40.96	23.72	0.24	1.39	48.24	12	1.04
	*E. dunnii*_winter_sim_pile1_sect3 s2b	5.14	40.85	23.67	0.25	1.52	48.29	11.89	1.04
	*E. dunnii*_winter_sim_pile2_sect1 s2	6.1	40.66	23.45	0.21	1.26	47.38	12.01	1.03
	*E. dunnii*_winter_sim_pile2_sect1 s2a	6.26	40.51	23.52	0.22	1.32	47.16	12.02	0.97
	*E. dunnii*_winter_sim_pile2_sect1 s2b	6.66	40.37	23.72	0.2	1.26	47.07	11.91	1.07
	*E. dunnii*_winter_sim_pile2_sect2 s2	5.3	40.6	24	0.23	1.43	47.91	12.05	1.03
	*E. dunnii*_winter_sim_pile2_sect2 s2a	5.33	41.01	23.85	0.23	1.35	48.22	12.09	1.07

	*E. dunnii*_winter_sim_pile2_sect2 s2b	5.04	40.26	24.11	0.22	1.44	47.61	12.14	1.05
	*E. dunnii*_winter_sim_pile2_sect3 s2	5.08	40.74	24.01	0.23	1.44	47.9	12.42	1.02
	*E. dunnii*_winter_sim_pile2_sect3 s2a	5.01	40.97	24.09	0.21	1.33	47.52	12.46	1.04
	*E. dunnii*_winter_sim_pile2_sect3 s2b	4.98	40.56	24.14	0.22	1.42	47.33	12.49	1.06
	*E. dunnii*_winter_sim_pile3_sect1 s2	5.22	40.57	24.31	0.22	1.36	48.19	12.16	1.06
	*E. dunnii*_winter_sim_pile3_sect1 s2a	5.45	40.88	24.12	0.21	1.24	47.93	12.25	1.02
	*E. dunnii*_winter_sim_pile3_sect1 s2b	5.11	40.51	23.98	0.23	1.45	48.17	12.18	1.08
	*E. dunnii*_winter_sim_pile3_sect2 s2	5.65	41.08	22.38	0.25	1.53	50.25	10.88	1.18
	*E. dunnii*_winter_sim_pile3_sect2 s2a	5.65	41.4	22.54	0.22	1.42	50	10.97	1.18
	*E. dunnii*_winter_sim_pile3_sect2 s2b	5.31	40.56	22.45	0.24	1.61	50.4	10.76	1.26
	*E. dunnii*_winter_sim_pile3_sect3 s2	4.42	40.99	23.53	0.25	1.47	48.7	12.29	1.1
	*E. dunnii*_winter_sim_pile3_sect3 s2a	4.85	41.04	23.29	0.25	1.41	48.83	12.11	1.09
	*E. dunnii*_winter_sim_pile3_sect3 s2b	4.5	40.75	23.5	0.24	1.52	48.63	12.11	1.15
Time 0	*E. dunnii*_time2	10.91	40.27	20.75	0.4	1.38	49.3	9.23	1.26
	*E. dunnii*_time2	11.86	40.69	19.98	0.41	1.39	49.2	9.05	1.34
	*E. dunnii*_time2	10.9	40.14	19.92	0.43	1.53	49.69	8.88	1.32
Summer (S1)	*E. dunnii*_summer_sim_pile1_sect1 s1_27.10.10	6.01	39.65	25.4	0.4	1.64	44.64	12.93	0.67
	*E. dunnii*_summer_sim_pile1_sect1s1_27.10.10a	6.18	39.33	25.31	0.38	1.65	44.72	12.85	0.67
	*E. dunnii*_summer_sim_pile1_sect1 s1 27.10.10b	5.94	39.52	25.26	0.39	1.68	44.68	12.87	0.69
	*E. dunnii*_summer_sim_pile1_sect2 s1_27.10.10	5.7	39.57	25.08	0.4	1.55	44.66	13.15	0.72
	*E. dunnii*_summer_sim_pile1_sect2 s1_27.10.10a	6.04	39.4	24.97	0.41	1.57	44.87	13.09	0.72
	*E .dunnii*_summer_sim_pile1_sect2 s1_27.10.10b	5.73	39.47	24.87	0.41	1.64	44.6	13.09	0.72
	*E. dunnii*_summer_sim_pile1_sect3 s1_27.10.10	6.31	39.37	25.54	0.39	1.52	44.26	13.36	0.69
	*E. dunnii*_summer_sim_pile1_sect3 s1_27.10.10a	6.07	39.37	25.33	0.39	1.58	44.13	13.33	0.73
	*E. dunnii*_summer_sim_pile1_sect3 s1_27.10.10b	5.97	39.55	25.46	0.39	1.56	44.42	13.33	0.77
	*E. dunnii*_summer_sim_pile2_sect1 s1_27.10.10	6.53	39.28	25.89	0.35	1.36	44.02	13.25	0.72
	*E. dunnii*_summer_sim_pile2_sect1 s1_27.10.10a	6.01	39.54	25.76	0.37	1.41	44.28	13.17	0.7
	*E. dunnii*_summer_sim_pile2_sect1 s1_27.10.10b	6.47	39.5	25.62	0.34	1.35	43.99	13.2	0.77
	*E. dunnii*_summer_sim_pile2_sect2 s1_27.10.10	5.23	39.89	24.48	0.42	1.72	45.5	12.84	0.73

	*E. dunnii*_summer_sim_pile2_sect2 s1_27.10.10a	5.47	39.79	24.55	0.42	1.7	45.34	12.62	0.73
	*E. dunnii*_summer_sim_pile2_sect2 s1_27.10.10b	5.32	39.64	24.46	0.42	1.73	45.53	12.74	0.76
	*E. dunnii*_summer_sim_pile2_sect3 s1_27.10.10	6.17	39.21	25.26	0.39	1.42	44.57	13.26	0.74
	*E. dunnii*_summer_sim_pile2_sect3 s1_27.10.10a	6.44	38.8	25.59	0.39	1.36	44.11	13.32	0.73
	*E. dunnii*_summer_sim_pile2_sect3 s1_27.10.10b	6.28	39.02	24.93	0.4	1.51	44.75	13.21	0.76
	*E. dunnii*_summer_sim_pile3_sect1 s1_27.10.10	5.66	40.08	24.95	0.4	1.71	45.2	12.79	0.6
	*E. dunnii*_summer_sim_pile3_sect1 s1_27.10.10a	5.88	39.97	25.35	0.4	1.62	44.77	12.75	0.58
	*E. dunnii*_summer_sim_pile3_sect1 s1_27.10.10b	5.69	39.79	25.08	0.4	1.76	45.03	12.66	0.63
	*E. dunnii*_summer_sim_pile3_sect2 s1_27.10.10	5.97	39.21	25.35	0.41	1.67	44.58	12.76	0.64
	*E. dunnii*_summer_sim_pile3_sect2 s1_27.10.10a	5.8	38.84	25.7	0.4	1.68	44.17	12.79	0.63
	*E. dunnii*_summer_sim_pile3_sect2 s1_27.10.10b	5.68	38.92	25.35	0.41	1.76	44.34	12.78	0.68
	*E. dunnii*_summer_sim_pile3_sect3 s1_27.10.10	4.49	39.43	25.17	0.47	1.78	44.77	13.06	0.57
	*E. dunnii*_summer_sim_pile3_sect3 s1_27.10.10a	5.92	39.16	24.93	0.44	1.75	44.71	12.96	0.6
	*E. dunnii*_summer_sim_pile3_sect3 s1_27.10.10b	5.27	39.41	24.87	0.45	1.74	44.79	12.9	0.6
Summer (S2)	*E. dunnii*_summer_sim_pile1_sect1 s2_10/11/10	5.78	39.77	24.85	0.39	1.5	44.96	13.07	0.76
	*E. dunnii*_summer_sim_pile1_sect1 s2_10/11/10a	5.88	39.95	24.9	0.38	1.48	44.91	13.13	0.79
	*E. dunnii*_summer_sim_pile1_sect1 s2_10/11/10b	5.43	39.64	25.03	0.36	1.44	44.79	13.06	0.85
	*E. dunnii*_summer_sim_pile2_sect1 s2_10/11/10	5.68	39.77	24.85	0.4	1.61	44.97	12.96	0.75
	*E. dunnii*_summer_sim_pile2_sect1 s2_10/11/10a	5.97	39.72	25.08	0.4	1.56	44.64	13.03	0.76
	*E. dunnii*_summer_sim_pile2_sect1 s2_10/11/10b	5.21	39.78	24.71	0.41	1.67	45.06	13.01	0.75
	*E. dunnii*_summer_sim_pile2_sect2 s2_10/11/10	6.34	39.68	25.4	0.38	1.49	44.45	12.95	0.73
	*E. dunnii*_summer_sim_pile2_sect2 s2_10/11/10a	5.95	39.71	25.36	0.39	1.47	44.64	13.04	0.72
	*E. dunnii*_summer_sim_pile2_sect2 s2_10/11/10b	6.38	39.48	25.23	0.4	1.54	44.48	13.04	0.72
	*E. dunnii*_summer_sim_pile2_sect3 s2_10/11/10	6.85	39.44	25.33	0.37	1.41	43.91	13.22	0.77
	*E. dunnii*_summer_sim_pile2_sect3 s2_10/11/10a	7.13	39.5	25.29	0.37	1.37	44.03	13.08	0.75
	*E. dunnii*_summer_sim_pile2_sect3 s2_10/11/10b	6.72	39.25	25.04	0.39	1.48	44.44	13.11	0.74
	*E. dunnii*_summer_sim_pile3_sect1 s2_10/11/10	5.61	40.21	24.93	0.37	1.53	45.03	13.01	0.83
	*E. dunnii*_summer_sim_pile3_sect1 s2_10/11/10a	6.19	39.96	25.05	0.38	1.53	44.91	13.01	0.76
	*E. dunnii*_summer_sim_pile3_sect1 s2_10/11/10b	5.74	39.86	25.01	0.38	1.53	45.08	13.01	0.8

	*E. dunnii*_summer_sim_pile3_sect2 s2_10/11/10	5.51	39.58	24.96	0.4	1.59	45.07	13.06	0.74
	*E. dunnii*_summer_sim_pile3_sect2 s2_10/11/10a	5.57	39.75	24.86	0.39	1.54	45.15	12.98	0.79
	*E. dunnii*_summer_sim_pile3_sect2 s2_10/11/10b	5.7	39.61	24.79	0.39	1.56	45.28	12.89	0.77
	*E. dunnii*_summer_sim_pile3_sect3 s2_10/11/10	6.45	40.22	25.18	0.36	1.56	44.89	12.93	0.78
	*E. dunnii*_summer_sim_pile3_sect3 s2_10/11/10a	6.59	39.96	25.19	0.36	1.55	44.68	13	0.73
	*E. dunnii*_summer_sim_pile3_sect3 s2_10/11/10b	6.62	39.69	25.25	0.36	1.58	44.59	12.98	0.76
	*E. dunnii*_summer_sim_pile1_sect2 s2_10/11/10	6.12	40.25	24.98	0.38	1.53	44.7	12.97	0.75
	*E. dunnii*_summer_sim_pile1_sect2 s2_10/11/10a	6.02	40.12	25.04	0.39	1.59	44.59	12.96	0.73
	*E. dunnii*_summer_sim_pile1_sect2 s2_10/11/10b	6	39.69	25.29	0.38	1.58	44.34	13.07	0.81
	*E. dunnii*_summer_sim_pile1_sect3 s2_10/11/10	6.72	40.23	24.76	0.39	1.72	44.89	12.74	0.72
	*E. dunnii*_summer_sim_pile1_sect3 s2_10/11/10a	6.66	40.15	24.92	0.39	1.72	44.65	12.69	0.75
	*E. dunnii*_summer_sim_pile1_sect3 s2_10/11/10b	6.52	40.08	24.88	0.38	1.72	44.72	12.71	0.74

Note: Time 0 = sample collected before start of experiment

S1 = sample collected after 2 weeks of simulation

S2 = sample collected after 4 weeks of simulation

Table A3: Chemistry of *E. nitens* chips in the simulated and control piles

	Samples	Hot water Extractives %	Cellulose %	Acid Insoluble Lignin %	Arabinose %	Galactose %	Glucose %	Xylose %	Mannose %
Time 0	*E. nitens*	3.59	45.2	24.3	0.1	0.63	51.45	11.37	1.5
	E. nitens a	3.34	45.27	24.31	0.09	0.65	51.61	11.32	1.57
	E. nitens b	3.25	45.25	24.29	0.1	0.66	51.44	11.37	1.55
Control Sappi (S1)	*E. nitens*_control_Sappi_Sect1 s1 21.09	1.78	43.44	24.59	0.16	1.12	46.46	13.89	1.18
	*E. nitens*_control_Sappi_Sect1 s1 21.09a	1.48	43.73	24.36	0.17	1.16	46.64	13.88	1.2
	*E. nitens*_control_Sappi_Sect1 s1 21.09b	1.55	43.77	24.23	0.17	1.17	46.78	13.85	1.21
	*E. nitens*_control_Sappi_Sect2 s1 21.09	5.43	43.34	23.39	0.18	0.82	49.43	11.48	1.41
	*E. nitens*_control_Sappi_Sect2 s1 21.09a	5.26	42.95	23.45	0.19	0.92	49.39	11.43	1.37
	*E. nitens*_control_Sappi_Sect2 s1 21.09b	5.26	42.8	23.27	0.19	0.96	49.56	11.33	1.41
	*E. nitens*_control_Sappi_Sect3 s1 21.09	3.53	42.52	24.29	0.23	1.3	46.73	13.04	1.15
	*E. nitens*_control_Sappi_Sect3 s1 21.09a	3.89	42.63	24.13	0.24	1.24	46.31	13.23	1.12
	*E. nitens*_control_Sappi_Sect3 s1 21.09b	3.27	42.35	24.06	0.24	1.32	46.43	13.08	1.17
Control Sappi (S2)	*E. nitens*_control_Sappi_sect1 s2_05.10.10	3.55	44.45	22.21	0.12	0.6	52.6	10.16	1.59
	*E. nitens*_control_Sappi_sect1 s2_05.10.10a	2.78	44.38	23.19	0.1	0.57	52.56	10.47	1.48
	*E. nitens*_control_Sappi_sect1 s2_05.10.10b	3.33	44.39	22.13	0.12	0.61	52.97	10.04	1.64
	*E. nitens*_control_Sappi_sect2 s2_05.10.10	2.84	43.44	23.64	0.14	0.88	49.16	11.81	1.34
	*E. nitens*_control_Sappi_sect2 s2_05.10.10a	2.95	43.85	23.27	0.14	0.95	49.56	11.51	1.37
	*E. nitens*_control_Sappi_sect2 s2_05.10.10b	2.69	43.52	23.54	0.14	0.91	49.35	11.81	1.36
	*E. nitens*_control_Sappi_sect3 s2_05.10.10	3.11	43.83	23.61	0.11	0.79	50.83	11.4	1.34
	*E. nitens*_control_Sappi_sect3 s2_05.10.10a	3	43.79	23.74	0.1	0.78	51.11	11.31	1.35
	*E. nitens*_control_Sappi_sect3 s2_05.10.10b	3.22	43.32	23.53	0.11	0.82	50.77	11.24	1.39
Control UKZN (S1)	*E. nitens*_control_UKZN_Sect1 s1 23.09	3.44	43.38	25.59	0.23	1.1	45.26	13.68	1.01
	*E. nitens*_control_UKZN_Sect1 s1 23.09a	3.01	42.53	25.51	0.25	1.36	45.33	13.66	1.05
	*E. nitens*_control_UKZN_Sect1 s1 23.09b	3.18	42.32	25.69	0.24	1.3	44.73	13.8	1.02
	*E. nitens*_control_UKZN_Sect2 s1 23.09	4.65	44.19	23.08	0.19	0.84	50.47	10.83	1.56

	*E. nitens*_control_UKZN_Sect2 s1 23.09a	4.38	44.35	23.27	0.17	0.81	50.76	10.84	1.56
	*E. nitens*_control_UKZN_Sect2 s1 23.09b	4.28	44.14	23.06	0.16	0.8	50.84	10.75	1.63
	*E. nitens*_control_UKZN_Sect3 s1 23.09	4.27	43.97	23.61	0.16	0.83	50.37	11.56	1.45
	*E. nitens*_control_UKZN_Sect3 s1 23.09a	4.37	43.76	23.72	0.15	0.75	50.13	11.68	1.48
	*E. nitens*_control_UKZN_Sect3 s1 23.09b	4.27	43.52	23.47	0.16	0.87	50.21	11.64	1.48
Control UKZN (S2)	*E. nitens*_control_UKZN_sect1 s2_07.10.10	7.26	42.55	21.24	0.27	0.8	49.15	9.95	1.74
	*E. nitens*_control_UKZN_sect1 s2_07.10.10a	5.92	43.2	23.53	0.21	0.67	48.27	10.89	1.52
	*E. nitens*_control_UKZN_sect1 s2_07.10.10b	7.16	43.15	21.6	0.24	0.79	48.68	10.22	1.71
	*E. nitens*_control_UKZN_sect2 s2_07.10.10	4.17	44.95	23.46	0.17	0.88	51.57	10.77	1.38
	*E. nitens*_control_UKZN_sect2 s2_07.10.10a	4.6	44.93	23.21	0.17	0.82	51.22	10.67	1.41
	*E. nitens*_control_UKZN_sect2 s2_07.10.10b	4.04	45.13	23.13	0.17	0.91	51.82	10.57	1.44
	*E. nitens*_control_UKZN_sect3 s2_07.10.10	3.66	45.37	23.65	0.14	0.64	51.64	10.96	1.48
	*E. nitens*_control_UKZN_sect3 s2_07.10.10a	3.74	45.35	23.37	0.15	0.68	51.9	10.89	1.46
	*E. nitens*_control_UKZN_sect3 s2_07.10.10b	3.68	45.2	23.48	0.15	0.66	51.96	10.95	1.47
Time 0	*E. nitens*	3.59	45.2	24.3	0.1	0.63	51.45	11.37	1.5
	E. nitens a	3.34	45.27	24.31	0.09	0.65	51.61	11.32	1.57
	E. nitens b	3.25	45.25	24.29	0.1	0.66	51.44	11.37	1.55
Winter (S1)	*E. nitens*_winter_sim_pile1_sect1 s1_29.09	4.12	43.89	25.41	0.23	0.87	47.35	12.09	1.25
	*E. nitens*_winter_sim_pile1_sect1 s1_29.09a	4.55	43.99	25.27	0.23	0.83	47.12	12.1	1.25
	*E. nitens*_winter_sim_pile1_sect1 s1_29.09b	3.89	43.91	25.34	0.24	0.9	47.8	12	1.26
	*E. nitens*_winter_sim_pile1_sect2 s1_29.09	4.91	42.63	25.81	0.25	0.81	47.17	11.89	1.42
	*E. nitens*_winter_sim_pile1_sect2 s1_29.09a	5.05	43.04	25.65	0.26	0.84	47.2	11.79	1.37
	*E. nitens*_winter_sim_pile1_sect2 s1_29.09b	5.02	42.17	25.71	0.27	0.91	46.42	11.95	1.41
	*E. nitens*_winter_sim_pile1_sect3 s1_29.09	4.46	42.98	25.56	0.28	0.96	46.46	12.61	1.15
	*E. nitens*_winter_sim_pile1_sect3 s1_29.09a	4.93	43.27	25.29	0.29	0.87	46.55	12.59	1.15
	*E. nitens*_winter_sim_pile1_sect3 s1_29.09b	5.16	42.53	25.32	0.31	1.01	45.85	12.72	1.12
	*E. nitens*_winter_sim_pile2_sect1 s1_29.09	4.98	43.33	28.03	0.34	1.21	44.76	11.98	0.82
	*E. nitens*_winter_sim_pile2_sect1 s1_29.09a	5.31	43.35	28.03	0.36	1.25	44.58	11.95	0.82
	*E. nitens*_winter_sim_pile2_sect1 s1_29.09b	5.24	43.21	28.02	0.36	1.22	44.95	11.9	0.84

	*E. nitens*_winter_sim_pile2_sect2 s1_29.09	8	42.84	23.27	0.26	0.56	48.43	10.5	1.6
	*E. nitens*_winter_sim_pile2_sect2 s1_29.09a	8.69	42.87	22.83	0.28	0.59	48.34	10.37	1.63
	*E. nitens*_winter_sim_pile2_sect2 s1_29.09b	8.67	42.69	22.57	0.29	0.63	48.63	10.21	1.66
	*E. nitens*_winter_sim_pile2_sect3 s1_29.09	5.55	43.98	24.46	0.18	0.66	49.22	11.34	1.44
	*E. nitens*_winter_sim_pile2_sect3 s1_29.09a	5.66	43.83	24.55	0.18	0.66	48.72	11.45	1.42
	*E. nitens*_winter_sim_pile2_sect3 s1_29.09b	5.17	43.4	24.81	0.2	0.78	49.09	11.37	1.43
	*E. nitens*_winter_sim_pile3_sect1 s1_29.09	4.83	43.13	25.2	0.22	0.91	46.2	12.5	1.26
	*E. nitens*_winter_sim_pile3_sect1 s1_29.09a	4.92	43.27	25.11	0.23	0.92	46.1	12.49	1.21
	*E. nitens*_winter_sim_pile3_sect1 s1_29.09b	4.52	42.8	25.09	0.23	0.94	46.13	12.62	1.2
	*E. nitens*_winter_sim_pile3_sect2 s1_29.09	4.35	44.02	25.15	0.25	0.95	46.98	12.17	1.19
	*E. nitens*_winter_sim_pile3_sect2 s1_29.09a	4.7	43.94	25.18	0.24	0.89	47	12.15	1.21
	*E. nitens*_winter_sim_pile3_sect2 s1_29.09b	4.13	43.64	25.33	0.26	0.97	47.16	12.21	1.16
	*E. nitens*_winter_sim_pile3_sect3 s1_29.09	5.34	43.57	25.32	0.25	0.83	46.41	12.18	1.2
	*E. nitens*_winter_sim_pile3_sect3 s1_29.09a	5.23	43.97	25.27	0.26	0.84	46.29	12.17	1.17
	*E. nitens*_winter_sim_pile3_sect3 s1_29.09b	4.96	43.39	25.55	0.26	0.94	46.44	12.2	1.19
Winter (S2)	*E. nitens*_winter_sim_pile1_sect1 s2 _13.10.10	3.3	43.66	27.02	0.22	0.7	46.22	13.04	1.24
	*E. nitens*_winter_sim_pile1_sect1 s2 _13.10.10a	3.14	43.81	27	0.21	0.68	46.25	13.03	1.25
	*E. nitens*_winter_sim_pile1_sect1 s2 _13.10.10b	3.2	43.66	26.87	0.22	0.76	46.3	12.95	1.27
	*E. nitens*_winter_sim_pile1_sect2 s2 _13.10.10	2.8	44.57	26.94	0.24	0.79	47.41	12.52	1.08
	*E. nitens*_winter_sim_pile1_sect2 s2 _13.10.10a	2.52	44.56	27	0.25	0.78	47.23	12.65	1.09
	*E. nitens*_winter_sim_pile1_sect2 s2 _13.10.10b	2.42	44.18	26.88	0.23	0.8	47.45	12.63	1.16
	*E. nitens*_winter_sim_pile1_sect3 s2 _13.10.10	3.49	44.44	26.67	0.2	0.62	46.41	12.93	1.17
	*E. nitens*_winter_sim_pile1_sect3 s2 _13.10.10a	3.34	44.01	26.69	0.21	0.71	46.74	12.95	1.16
	*E. nitens*_winter_sim_pile1_sect3 s2 _13.10.10b	3.41	43.92	26.51	0.22	0.78	46.8	12.81	1.16
	*E. nitens*_winter_sim_pile2_sect1 s2 _13.10.10	3.02	44.96	26.81	0.22	0.66	47.39	12.58	1.14
	*E. nitens*_winter_sim_pile2_sect1 s2 _13.10.10a	3.12	45	26.8	0.22	0.62	47.29	12.61	1.13
	*E. nitens*_winter_sim_pile2_sect1 s2 _13.10.10b	2.88	44.8	26.73	0.21	0.68	47.31	12.65	1.16
	*E. nitens*_winter_sim_pile2_sect2 s2 _13.10.10	3.49	43.94	27.01	0.2	0.66	47.06	12.66	1.17
	*E. nitens*_winter_sim_pile2_sect2 s2 _13.10.10a	3.33	43.88	27.01	0.2	0.72	47.07	12.6	1.2

	*E. nitens*_winter_sim_pile2_sect2 s2 _13.10.10b	3.23	43.79	26.68	0.22	0.8	47.11	12.65	1.21
	*E. nitens*_winter_sim_pile2_sect3 s2 _13.10.10	3.16	44.02	26.62	0.2	0.62	46.59	12.98	1.21
	*E. nitens*_winter_sim_pile2_sect3 s2 _13.10.10a	3.25	44.08	26.72	0.22	0.61	46.63	12.97	1.18
	*E. nitens*_winter_sim_pile2_sect3 s2 _13.10.10b	3.1	43.5	26.64	0.22	0.73	46.52	12.97	1.25
	*E. nitens*_winter_sim_pile3_sect1 s2 _13.10.10	2.51	44.62	26.55	0.2	0.76	47.25	12.78	1.17
	*E. nitens*_winter_sim_pile3_sect1 s2 _13.10.10a	2.48	44.65	26.73	0.21	0.67	46.86	12.95	1.18
	*E. nitens*_winter_sim_pile3_sect1 s2 _13.10.10b	2.86	44.37	26.62	0.2	0.68	47.05	12.81	1.21
	*E. nitens*_winter_sim_pile3_sect2 s2 _13.10.10	3.19	43.85	26.93	0.23	0.79	46.71	12.82	1.11
	*E. nitens*_winter_sim_pile3_sect2 s2 _13.10.10a	3.05	44.11	26.84	0.23	0.82	46.77	12.73	1.12
	*E. nitens*_winter_sim_pile3_sect2 s2 _13.10.10b	3.16	43.84	26.92	0.21	0.79	47	12.73	1.17
	*E. nitens*_winter_sim_pile3_sect3 s2 _13.10.10	2.85	44.05	26.64	0.23	0.82	46.82	12.96	1.16
	*E. nitens*_winter_sim_pile3_sect3 s2 _13.10.10a	2.85	44.1	26.83	0.23	0.79	46.71	12.91	1.16
	*E. nitens*_winter_sim_pile3_sect3 s2 _13.10.10b	2.78	44.31	26.64	0.23	0.79	47.01	12.82	1.19
Time 0	*E. nitens*_time1	6.59	44.16	23.28	0.16	0.37	50.35	9.85	1.69
	*E. nitens*_time1	6.51	44.18	23.22	0.16	0.35	50.28	9.9	1.72
	*E. nitens*_time1	6.21	44.13	23.34	0.17	0.39	50.48	9.85	1.68
Summer (S1)	*E. nitens*_summer_sim_pile1_sect1 s1_28.10.10	2.47	44.16	27.81	0.26	0.75	46.03	12.95	1.06
	*E. nitens*_summer_sim_pile1_sect1 s1_28.10.10a	2.64	44.03	27.49	0.25	0.83	46.04	12.94	1.07
	*E. nitens*_summer_sim_pile1_sect1 s1_28.10.10b	2.26	43.98	27.71	0.26	0.83	46.45	12.91	1.12
	*E. nitens*_summer_sim_pile1_sect2 s1_28.10.10	2.45	44.39	26.46	0.24	0.83	45.86	13.54	1.05
	*E. nitens*_summer_sim_pile1_sect2 s1_28.10.10a	2.33	44.1	26.28	0.25	0.96	46.02	13.58	1.04
	*E. nitens*_summer_sim_pile1_sect2 s1_28.10.10b	2.07	44.11	26.4	0.24	0.88	46.17	13.52	1.12
	*E. nitens*_summer_sim_pile1_sect3 s1_28.10.10	2	45.3	27.46	0.22	0.73	46.58	12.87	1.03
	*E. nitens*_summer_sim_pile1_sect3 s1_28.10.10a	1.91	44.63	27.64	0.24	0.85	46.36	12.93	1.02
	*E. nitens*_summer_sim_pile1_sect3 s1_28.10.10b	1.99	44.41	27.52	0.23	0.84	46.31	13.05	1.12
	*E. nitens*_summer_sim_pile2_sect1 s1_28.10.10	2.55	45.54	26.37	0.21	0.71	47.03	12.88	1.11
	*E. nitens*_summer_sim_pile2_sect1 s1_28.10.10a	2.47	45.28	26.55	0.22	0.63	47.12	12.92	1.09
	*E. nitens*_summer_sim_pile2_sect1 s1_28.10.10b	2.62	44.92	26.54	0.22	0.69	47.03	12.98	1.14
	*E. nitens*_summer_sim_pile2_sect2 s1_28.10.10	2.68	43.99	27.26	0.22	0.8	46.16	12.93	1.13

	*E. nitens*_summer_sim_pile2_sect2 s1_28.10.10a	1.9	43.75	27.55	0.25	0.86	46.28	12.96	1.1
	*E. nitens*_summer_sim_pile2_sect2 s1_28.10.10b	3.09	43.55	27.37	0.22	0.84	46.01	12.88	1.17
	*E. nitens*_summer_sim_pile2_sect3 s1_28.10.10	1.95	45.07	26.78	0.23	0.74	47.04	13.01	1.1
	*E. nitens*_summer_sim_pile2_sect3 s1_28.10.10a	2.61	44.83	26.73	0.22	0.69	46.74	13.01	1.13
	*E. nitens*_summer_sim_pile2_sect3 s1_28.10.10b	1.88	44.7	26.79	0.24	0.76	47.11	13.04	1.1
	*E. nitens*_summer_sim_pile3_sect1 s1_28.10.10	2.46	44.36	27.53	0.25	0.81	45.32	13.49	1.07
	*E. nitens*_summer_sim_pile3_sect1 s1_28.10.10a	2.11	43.94	27.64	0.22	0.75	45.15	13.53	1.15
	*E. nitens*_summer_sim_pile3_sect1 s1_28.10.10b	2.54	43.67	27.87	0.24	0.85	44.99	13.51	1.1
	*E. nitens*_summer_sim_pile3_sect2 s1_28.10.10	2.46	44.78	26.64	0.23	0.86	46.72	12.86	1.05
	*E. nitens*_summer_sim_pile3_sect2 s1_28.10.10a	1.93	44.46	26.61	0.25	0.91	46.92	12.89	1.05
	*E. nitens*_summer_sim_pile3_sect2 s1_28.10.10b	2.1	44.31	26.66	0.26	1.01	46.71	12.83	1.07
	*E. nitens*_summer_sim_pile3_sect3 s1_28.10.10	1.46	43.84	27.77	0.3	0.99	46.18	12.9	1.04
	*E. nitens*_summer_sim_pile3_sect3 s1_28.10.10a	1.58	43.87	28	0.29	1.03	46.07	12.76	1.04
	*E. nitens*_summer_sim_pile3_sect3 s1_28.10.10b	1.58	43.91	27.73	0.29	0.99	46.42	12.8	1.06
Summer (S2)	*E. nitens*_summer_sim_pile1_sect1 s2_11/11/10	2.04	44.57	25.98	0.23	1.04	46.83	13	1.06
	*E. nitens*_summer_sim_pile1_sect1 s2_11/11/10a	1.94	45.04	25.85	0.21	0.94	47.26	12.9	1.07
	*E. nitens*_summer_sim_pile1_sect1 s2_11/11/10b	2.67	44.33	26.15	0.27	1.08	46.34	12.88	1.03
	*E. nitens*_summer_sim_pile1_sect2 s2_11/11/10	3.62	43.63	26.81	0.3	0.87	44.73	13.43	1.1
	*E. nitens*_summer_sim_pile1_sect2 s2_11/11/10a	3.05	43.31	27.04	0.32	0.94	44.75	13.39	1.05
	*E. nitens*_summer_sim_pile1_sect2 s2_11/11/10b	3.35	42.83	26.67	0.33	1.05	44.77	13.43	1.07
	*E. nitens*_summer_sim_pile1_sect3 s2_11/11/10	4.17	43.61	26.2	0.32	0.94	44.81	13.44	1.02
	*E. nitens*_summer_sim_pile1_sect3 s2_11/11/10a	3.36	43.97	26.16	0.33	1.01	44.87	13.44	1.02
	*E. nitens*_summer_sim_pile1_sect3 s2_11/11/10b	3.83	43.67	26.3	0.33	1	44.88	13.38	1.04
	*E. nitens*_summer_sim_pile2_sect1 s2_11/11/10	3.84	43.29	27.06	0.36	1.08	44.37	13.2	0.98
	*E. nitens*_summer_sim_pile2_sect1 s2_11/11/10a	4.39	42.89	27.06	0.36	1.07	44.1	13.33	1.01
	*E. nitens*_summer_sim_pile2_sect1 s2_11/11/10b	3.99	43.03	27.1	0.36	1.08	44.23	13.24	0.97
	*E. nitens*_summer_sim_pile2_sect2 s2_11/11/10	4.85	43.26	27.16	0.33	1.01	44.14	12.99	1.02
	*E. nitens*_summer_sim_pile2_sect2 s2_11/11/10a	4.78	42.87	27.47	0.36	1.05	44.25	13.01	0.9
	*E. nitens*_summer_sim_pile2_sect2 s2_11/11/10b	4.31	43.26	27.38	0.37	1.13	44.24	12.96	0.95

	*E. nitens*_summer_sim_pile2_sect3 s2_11/11/10	4.63	43.41	26.83	0.34	1.04	43.91	13.25	1
	*E. nitens*_summer_sim_pile2_sect3 s2_11/11/10a	4.72	43.26	27	0.36	1.04	43.89	13.34	1
	*E. nitens*_summer_sim_pile2_sect3 s2_11/11/10b	4.45	42.94	27.18	0.35	1.03	43.94	13.24	0.98
	*E. nitens*_summer_sim_pile3_sect1 s2_11/11/10	2.51	44.15	26.31	0.22	0.76	45.22	13.92	1.16
	*E. nitens*_summer_sim_pile3_sect1 s2_11/11/10a	2.14	44.08	26.28	0.22	0.81	45.57	13.87	1.15
	*E. nitens*_summer_sim_pile3_sect1 s2_11/11/10b	1.93	44.01	26.03	0.22	0.86	45.66	13.85	1.21
	*E. nitens*_summer_sim_pile3_sect2 s2_11/11/10	2.79	44.62	25.45	0.21	1	46.32	12.93	1.23
	*E. nitens*_summer_sim_pile3_sect2 s2_11/11/10a	2.63	44.58	25.56	0.21	1.01	46.1	12.96	1.2
	*E. nitens*_summer_sim_pile3_sect2 s2_11/11/10b	2.08	44.57	25.44	0.2	1.07	46.74	12.87	1.25
	*E. nitens*_summer_sim_pile3_sect3 s2_11/11/10	1.15	45.78	26.12	0.18	0.89	47.81	12.81	1.14
	*E. nitens*_summer_sim_pile3_sect3 s2_11/11/10a	1.16	45.58	26.27	0.15	0.83	47.93	12.73	1.17
	*E. nitens*_summer_sim_pile3_sect3 s2_11/11/10b	1.17	45.42	26.27	0.16	0.81	48.09	12.79	1.2

Note: Time 0 = sample collected before start of experiment

S1 = sample collected after 2 weeks of simulation

S2 = sample collected after 4 weeks of simulation

Table A4: Chemistry of wood chips from the combination simulations and control piles

	Samples	Hot water Extractives %	Cellulose %	Acid Insoluble Lignin %	Arabinose %	Galactose %	Glucose %	Xylose %	Mannose %
Time 0	Combination Time zero	6.80	42.01	22.02	0.28	1.12	49.26	10.98	1.49
	Combination Time zero a	6.76	41.68	22.26	0.30	1.22	49.27	11.80	1.45
	Combination Time zero b	6.69	41.63	22.52	0.29	1.15	49.05	11.11	1.43
Winter (S1)	Combination1_Winter_s1_Pile1_Sec1_22/02/11	3.94	41.69	24.56	0.29	1.24	45.27	13.53	1.18
	Combination1_Winter_s1_Pile1_Sec1_22/02/11a	3.82	41.19	24.65	0.3	1.31	45.57	13.57	1.23
	Combination1_Winter_s1_Pile1_Sec1_22/02/11b	3.43	41.55	24.5	0.29	1.27	45.53	13.57	1.23
	Combination1_Winter_s1_Pile1_Sec2_22/02/11	5.61	41.46	23.65	0.28	1.1	49.59	10.12	1.41
	Combination1_Winter_s1_Pile1_Sec2_22/02/11a	5.54	41.3	23.77	0.28	1.12	49.55	10.13	1.41
	Combination1_Winter_s1_Pile1_Sec2_22/02/11b	5.42	41.48	23.56	0.28	1.13	49.61	10.09	1.41
	Combination1_Winter_s1_Pile1_Sec3_22/02/11	3.51	42.8	25.18	0.27	1.13	46.16	12.81	1.12
	Combination1_Winter_s1_Pile1_Sec3_22/02/11a	3.66	42.68	25.34	0.28	1.12	46.17	12.83	1.13
	Combination1_Winter_s1_Pile1_Sec3_22/02/11b	3.69	42.71	25.37	0.27	1.11	46.2	12.8	1.17
	Combination1_Winter_s1_Pile2_Sec1_22/02/11	3.55	42.82	25.05	0.26	1.21	46.03	12.87	1.14
	Combination1_Winter_s1_Pile2_Sec1_22/02/11a	3.34	42.61	24.96	0.29	1.33	46.17	12.96	1.14
	Combination1_Winter_s1_Pile2_Sec1_22/02/11b	3.36	42.86	24.67	0.28	1.24	46.3	12.94	1.18
	Combination1_Winter_s1_Pile2_Sec2_22/02/11	5.72	41.96	23.46	0.28	1.03	49	10.63	1.33
	Combination1_Winter_s1_Pile2_Sec2_22/02/11a	5.75	41.66	23.56	0.28	1.06	49.19	10.48	1.35
	Combination1_Winter_s1_Pile2_Sec2_22/02/11b	5.82	41.8	23.33	0.29	1.18	48.92	10.48	1.36
	Combination1_Winter_s1_Pile2_Sec3_22/02/11	5.81	42.14	24.42	0.25	1	47.02	12.1	1.24
	Combination1_Winter_s1_Pile2_Sec3_22/02/11a	5.57	41.89	24.49	0.25	1	47.14	12.13	1.24
	Combination1_Winter_s1_Pile2_Sec3_22/02/11b	5.22	41.38	24.94	0.27	1.06	47.67	12.15	1.23
	Combination1_Winter_s1_Pile3_Sec1_22/02/11	3.58	42.61	24.4	0.27	1.12	45.33	13.67	1.1
	Combination1_Winter_s1_Pile3_Sec1_22/02/11a	3.49	42.51	24.35	0.28	1.15	45.56	13.52	1.16
	Combination1_Winter_s1_Pile3_Sec1_22/02/11b	3.41	42.43	24.48	0.28	1.17	45.71	13.61	1.16
	Combination1_Winter_s1_Pile3_Sec2_22/02/11	4.26	41.66	24.38	0.3	1.24	46.47	12.81	1.11

	Combination1_Winter_s1_Pile3_Sec2_22/02/11a	4.3	42.14	24.03	0.29	1.22	46.57	12.71	1.15
	Combination1_Winter_s1_Pile3_Sec2_22/02/11b	4.36	41.87	24.03	0.29	1.26	46.46	12.74	1.14
	Combination1_Winter_s1_Pile3_Sec3_22/02/11	3.91	43.02	25.17	0.26	1	45.92	12.98	1.13
	Combination1_Winter_s1_Pile3_Sec3_22/02/11a	3.78	43.12	25.33	0.25	0.99	46.01	12.8	1.19
	Combination1_Winter_s1_Pile3_Sec3_22/02/11b	3.33	43.16	25.51	0.23	0.97	46.03	12.88	1.23
Winter (S2)	Combination1_Winter_s2_Pile1_Sec1_08/03/11	3.57	42.85	23.66	0.3	1.29	44.51	13.82	1.16
	Combination1_Winter_s2_Pile1_Sec1_08/03/11a	3.39	42.2	24.02	0.29	1.28	44.85	13.77	1.24
	Combination1_Winter_s2_Pile1_Sec1_08/03/11b	2.79	42.16	24.31	0.3	1.33	44.97	13.88	1.23
	Combination1_Winter_s2_Pile1_Sec2_08/03/11	4.49	42.23	23.49	0.3	1.41	46.24	12.36	1.07
	Combination1_Winter_s2_Pile1_Sec2_08/03/11a	4.36	42.16	23.78	0.29	1.36	46.35	12.36	1.12
	Combination1_Winter_s2_Pile1_Sec2_08/03/11b	4.16	42.04	23.79	0.3	1.38	46.7	12.35	1.14
	Combination1_Winter_s2_Pile1_Sec3_08/03/11	4.08	42.34	24.27	0.29	1.19	45.86	12.73	1.1
	Combination1_Winter_s2_Pile1_Sec3_08/03/11a	2.97	42.42	24.92	0.23	1.04	45.36	12.8	1.31
	Combination1_Winter_s2_Pile1_Sec3_08/03/11b	2.68	42.3	24.59	0.31	1.33	45.61	13.11	1.22
	Combination1_Winter_s2_Pile2_Sec1_08/03/11	4.21	42.6	23.41	0.3	1.26	45.19	13.14	1.11
	Combination1_Winter_s2_Pile2_Sec1_08/03/11a	3.16	43.11	23.15	0.33	1.44	44.82	13.49	1.1
	Combination1_Winter_s2_Pile2_Sec1_08/03/11b	4.2	42.17	23.85	0.3	1.19	45.73	13.1	1.18
	Combination1_Winter_s2_Pile2_Sec2_08/03/11	4.48	42.27	23.49	0.32	1.4	45.81	12.59	1.08
	Combination1_Winter_s2_Pile2_Sec2_08/03/11a	4.89	41.52	23.75	0.34	1.39	45.83	12.6	1.06
	Combination1_Winter_s2_Pile2_Sec2_08/03/11b	4.31	41.52	24.16	0.31	1.32	46.24	12.51	1.16
	Combination1_Winter_s2_Pile2_Sec3_08/03/11	3.74	41.96	24.79	0.3	1.3	45.18	12.98	1.1
	Combination1_Winter_s2_Pile2_Sec3_08/03/11a	3.64	42.42	24.69	0.29	1.25	45.07	12.94	1.17
	Combination1_Winter_s2_Pile2_Sec3_08/03/11b	4.32	42.03	24.87	0.28	1.14	45.62	12.67	1.19
	Combination1_Winter_s2_Pile3_Sec1_08/03/11	5.58	41.9	24.14	0.21	0.7	46.95	12.45	1.23
	Combination1_Winter_s2_Pile3_Sec1_08/03/11a	5.72	41.08	25	0.14	0.48	47.09	12.22	1.36
	Combination1_Winter_s2_Pile3_Sec1_08/03/11b	5.39	40.9	25.24	0.14	0.52	47.19	12.24	1.39
	Combination1_Winter_s2_Pile3_Sec2_08/03/11	5.79	40.46	24.77	0.16	0.59	46.28	12.31	1.28
	Combination1_Winter_s2_Pile3_Sec2_08/03/11a	5.67	40.14	24.93	0.21	0.74	46.91	12.34	1.25
	Combination1_Winter_s2_Pile3_Sec2_08/03/11b	6.14	40.26	25.02	0.18	0.58	47	12.24	1.29

	Combination1_Winter_s2_Pile3_Sec3_08/03/11	6.24	40.38	25.49	0.13	0.53	46.17	12.24	1.32
	Combination1_Winter_s2_Pile3_Sec3_08/03/11a	5.89	40.98	24.79	0.2	0.83	46.13	12.34	1.17
	Combination1_Winter_s2_Pile3_Sec3_08/03/11b	6.02	40.22	25.64	0.14	0.57	46.34	12.16	1.34
Time 0	Combination Time zero	7.58	41.66	23.36	0.32	1.11	48.83	10.57	1.32
	Combination Time zero a	8.18	41.79	22.96	0.33	1.15	48.75	10.45	1.37
	Combination Time zero b	7.75	41.47	22.91	0.34	1.19	48.9	10.37	1.35
Summer (S1)	Combination 1_summer simulation_pile1_sec1_25/01/11	3.07	43.04	25.81	0.25	1.04	46.52	13.02	1.17
	Combination 1_summer simulation_pile1_sec1_25/01/11a	2.53	42.69	26.09	0.26	1.17	46.85	12.97	1.2
	Combination 1_summer simulation_pile1_sec1_25/01/11b	2.76	42.81	25.85	0.25	1.14	46.69	12.98	1.21
	Combination 1_summer simulation _pile1_sec2_25/01/11	4.15	42.43	24.53	0.25	0.94	50.16	10.78	1.37
	Combination 1_summer simulation _pile1_sec2_25/01/11a	4.31	42.05	24.45	0.25	0.98	50.4	10.72	1.42
	Combination 1_summer simulation _pile1_sec2_25/01/11b	4.06	42.4	24.26	0.25	1.02	50.36	10.65	1.4
	Combination 1_summer simulation _pile1_sec3_25/01/11	3.76	41.73	25.43	0.28	1.28	48.4	11.98	1.17
	Combination 1_summer simulation _pile1_sec3_25/01/11a	3.89	42.24	25.21	0.27	1.23	48.2	12.03	1.16
	Combination 1_summer simulation _pile1_sec3_25/01/11b	3.57	42.15	25.04	0.29	1.36	48.42	11.94	1.14
	Combination 1_summer simulation _pile2_sec1_25/01/11	2.78	41.84	26.39	0.28	1.35	47.2	12.74	1.12
	Combination 1_summer simulation _pile2_sec1_25/01/11a	3.46	42.18	25.68	0.29	1.29	47.63	12.43	1.15
	Combination 1_summer simulation _pile2_sec1_25/01/11b	3.47	41.82	26	0.28	1.28	47.27	12.69	1.12
	Combination 1_summer simulation _pile2_sec2_25/01/11	4.67	41.61	25.32	0.24	1.07	48.73	10.99	1.36
	Combination 1_summer simulation _pile2_sec2_25/01/11a	4.4	41.71	25.18	0.24	1.13	48.93	10.92	1.36
	Combination 1_summer simulation _pile2_sec2_25/01/11b	4.49	41.43	25.14	0.24	1.15	48.71	10.97	1.38
	Combination 1_summer simulation _pile2_sec3_25/01/11	3.46	41.49	25.67	0.31	1.29	47.11	12.72	1.12
	Combination 1_summer simulation _pile2_sec3_25/01/11a	2.7	41.38	25.9	0.3	1.37	47.43	12.74	1.16
	Combination 1_summer simulation _pile2_sec3_25/01/11b	3.13	41.33	25.88	0.3	1.29	47.23	12.78	1.17
	Combination 1_summer simulation _pile3_sec1_25/01/11	3.06	41.83	26.16	0.26	1.28	45.93	13.16	1.11
	Combination 1_summer simulation _pile3_sec1_25/01/11a	3.21	41.58	26.1	0.27	1.27	45.99	13.13	1.12
	Combination 1_summer simulation _pile3_sec1_25/01/11b	3.35	41.91	25.91	0.26	1.2	45.92	13.12	1.14
	Combination 1_summer simulation _pile3_sec2_25/01/11	3.26	41.69	25.57	0.28	1.19	46.37	13.05	1.09
	Combination 1_summer simulation _pile3_sec2_25/01/11a	3.45	41.87	25.43	0.28	1.25	46.57	12.96	1.12

	Combination 1_summer simulation _pile3_sec2_25/01/11b	2.98	41.58	25.81	0.29	1.24	46.66	13.07	1.13
	Combination 1_summer simulation _pile3_sec3_25/01/11	3.29	41.95	25.3	0.3	1.43	46.04	13.19	1.04
	Combination 1_summer simulation _pile3_sec3_25/01/11a	3.21	42.02	25.36	0.31	1.47	46.5	13.1	1.07
	Combination 1_summer simulation _pile3_sec3_25/01/11b	3.18	42.15	25.34	0.31	1.47	46.49	13.06	1.05
Summer (S2)	Combination1_Summer_s2_Pile1_Sec1_08/02/11	3.47	42.73	24.69	0.28	1.17	46.54	12.88	1.12
	Combination1_Summer_s2_Pile1_Sec1_08/02/11a	3.48	42.24	24.68	0.27	1.21	45.92	12.91	1.19
	Combination1_Summer_s2_Pile1_Sec1_08/02/11b	3.42	42.61	24.49	0.29	1.29	46.41	12.84	1.15
	Combination1_Summer_s2_Pile1_Sec2_08/02/11	3.39	41.78	24.94	0.31	1.34	46.33	12.84	1.03
	Combination1_Summer_s2_Pile1_Sec2_08/02/11a	3.02	41.75	25.03	0.32	1.38	46.54	12.79	1.04
	Combination1_Summer_s2_Pile1_Sec2_08/02/11b	2.86	41.45	25.18	0.32	1.48	46.56	12.84	1.05
	Combination1_Summer_s2_Pile1_Sec3_08/02/11	3.6	42.3	25.6	0.28	1.18	46.2	12.66	1.11
	Combination1_Summer_s2_Pile1_Sec3_08/02/11a	3.79	42.67	25.19	0.31	1.24	46.22	12.63	1.08
	Combination1_Summer_s2_Pile1_Sec3_08/02/11b	3.32	41.07	25.66	0.31	1.41	45.65	12.71	1.16
	Combination1_Summer_s2_Pile2_Sec1_08/02/11	3.76	42.99	25.11	0.28	1.21	46.4	12.39	1.12
	Combination1_Summer_s2_Pile2_Sec1_08/02/11a	3.25	42.26	25.51	0.3	1.24	46.61	12.58	1.05
	Combination1_Summer_s2_Pile2_Sec1_08/02/11b	3.56	42.57	25.02	0.29	1.29	46.15	12.5	1.17
	Combination1_Summer_s2_Pile2_Sec2_08/02/11	3.04	42.2	25.31	0.31	1.34	46.5	12.74	1.04
	Combination1_Summer_s2_Pile2_Sec2_08/02/11a	3.05	42.21	25.26	0.31	1.34	46.59	12.73	1.06
	Combination1_Summer_s2_Pile2_Sec2_08/02/11b	2.57	41.32	25.24	0.33	1.59	46.42	12.74	1.11
	Combination1_Summer_s2_Pile2_Sec3_08/02/11	4.33	41.18	25.35	0.28	1.19	45.38	12.99	1.05
	Combination1_Summer_s2_Pile2_Sec3_08/02/11a	4.25	41.43	25.31	0.29	1.2	45.51	12.85	1.08
	Combination1_Summer_s2_Pile2_Sec3_08/02/11b	3.98	40.98	25.19	0.29	1.34	44.93	12.87	1.14
	Combination1_Summer_s2_Pile3_Sec1_08/02/11	3.57	41.39	24.48	0.35	1.62	46.66	12.64	0.96
	Combination1_Summer_s2_Pile3_Sec1_08/02/11a	3.72	41.25	24.61	0.34	1.64	46.63	12.77	0.97
	Combination1_Summer_s2_Pile3_Sec1_08/02/11b	3.53	41.14	24.49	0.32	1.56	46.65	12.63	1.03
	Combination1_Summer_s2_Pile3_Sec2_08/02/11	4.06	41.86	24.94	0.28	1.18	46.07	12.79	1.13
	Combination1_Summer_s2_Pile3_Sec2_08/02/11a	4.13	41.55	24.83	0.29	1.28	45.76	12.87	1.11
	Combination1_Summer_s2_Pile3_Sec2_08/02/11b	3.92	41.54	24.79	0.3	1.28	45.67	12.89	1.12
	Combination1_Summer_s2_Pile3_sec3_08/02/11	5.13	41.47	24.61	0.24	1.07	46.96	12.3	1.12

	Combination1_Summer_s2_Pile3_sec3_08/02/11a	5.31	41.09	24.66	0.25	1.05	46.8	12.32	1.18
	Combination1_Summer_s2_Pile3_sec3_08/02/11b	4.71	41.73	24.8	0.22	0.96	47.12	12.28	1.29

APPENDIX B: PULPING DATA

Table B1: Pulping data for simulated and control *E. dunnii* chip piles

Sample ID	SPY	Viscosity	K - number	S10	S18	S10 - S18	Alpha cellulose	Copper number	Glucose (%)	Xylose (%)	Mannose (%)	Sum (%)	Klason Lignin (%)	Acid soluble lignin (%)	Total lignin (%)
E. dunnii - Time zero pulping - R1	50.51	28.08	4.01	9.72	7.06	2.66	91.61	3.14	90.60	2.94	1.20	94.71	2.27	1.69	3.96
E. dunnii - Time zero pulping - R2	49.89	27.88	4.12	9.84	7.10	2.74	91.53	3.16	90.95	2.70	1.78	95.42	2.20	1.69	3.89
E. dunnii - Time zero pulping - R3	48.65	28.04	4.05	9.75	7.09	2.66	91.54	3.15	90.79	2.8	1.5	95	2.25	1.68	3.94
E. dunnii - Control/UKZN - R1	37.25	51.97	4.58	10.98	7.98	3.01	90.52	3.14	87.08	5.72	0.81	93.61	3.04	2.11	5.15
E. dunnii - Control/UKZN - R2	38.48	54.55	4.51	10.88	8.09	2.79	90.52	3.12	91.12	6.56	0.70	98.38	2.92	1.76	4.68
E. dunnii - Control/UKZN - R3	39.07	43.15	4.44	10.90	7.92	2.98	90.59	3.16	88.70	6.07	0.76	95.53	3.24	1.68	4.92
E. dunnii - Control/Sappi - R1	42.9	48.30	4.40	11.02	8.18	2.85	90.40	3.04	84.82	4.96	0.62	90.40	2.76	1.56	4.32
E. dunnii- Control/Sappi - R2	43.8	52.27	4.33	10.98	8.12	2.87	90.45	3.05	83.97	4.93	0.60	89.50	2.64	1.68	4.32
E. dunnii- Control/Sappi - R3	41.85	50.29	4.22	10.29	8.05	2.24	90.83	3.02	84.15	5.82	0.92	90.89	3.05	1.66	4.70
E. dunnii - Summer Simulation - Pile 1 - R1	50.2	63.00	4.40	10.77	7.77	3.00	90.73	2.74	87.71	5.69	0.92	94.43	2.80	1.97	4.77
E. dunnii - Summer Simulation - Pile 1 - R2	51.63	60.26	4.72	10.84	7.96	2.89	90.60	2.74	83.64	5.49	0.75	89.99	2.79	1.25	4.04
E. dunnii - Summer Simulation - Pile 1 - R3	50.55	68.42	4.62	10.44	7.84	2.60	90.86	2.68	87.23	5.77	0.80	93.91	2.77	1.78	4.55
E. dunnii - Summer Simulation - Pile 2 - R1	44.87	74.66	4.58	11.02	7.95	3.07	90.52	2.70	86.70	5.85	0.72	93.37	2.95	1.90	4.85
E. dunnii - Summer Simulation - Pile 2 - R2	44.45	68.42	4.65	10.82	8.04	2.78	90.57	2.72	86.86	5.52	0.78	93.28	2.89	1.40	4.30
E. dunnii - Summer Simulation - Pile 2 - R3	45.44	75.69	4.69	10.80	7.91	2.90	90.65	2.71	86.62	6.41	0.82	93.95	3.11	1.96	5.07
E. dunnii - Summer Simulation - Pile 3 - R1	44.95	62.32	4.05	11.17	8.08	3.09	90.37	2.75	86.56	5.73	0.98	93.36	2.57	1.63	4.20
E. dunnii - Summer Simulation - Pile 3 - R2	44.87	55.74	3.91	11.11	8.21	2.90	90.34	2.82	86.85	5.66	0.97	93.58	2.68	1.77	4.45
E. dunnii - Summer Simulation - Pile 3 - R3	45.11	61.84	3.80	11.10	8.01	3.09	90.44	2.78	87.44	5.78	0.97	94.30	2.48	1.86	4.34
E. dunnii - Winter Simulation - Pile 1 - R1	41.8	88.75	5.72	11.14	8.47	2.67	90.19	3.19	83.15	6.54	0.52	90.21	3.76	1.77	5.53
E. dunnii - Winter Simulation - Pile 1 - R2	41.99	85.64	6.04	11.19	8.56	2.63	90.13	3.16	84.23	6.38	0.61	91.22	3.60	1.75	5.35

E. dunnii - Winter Simulation - Pile 1 - R3	40.99	72.11	5.86	11.16	8.53	2.62	90.16	3.16	83.57	6.39	0.69	90.65	4.00	1.67	5.68
E. dunnii - Winter Simulation - Pile 2 - R1	39.15	79.01	5.33	10.88	8.60	2.28	90.26	2.97	84.69	6.06	0.61	91.36	3.04	1.55	4.58
E. dunnii - Winter Simulation - Pile 2 - R2	38.81	77.28	5.08	10.99	8.54	2.45	90.24	2.95	83.63	6.28	0.58	90.48	2.91	1.81	4.73
E. dunnii - Winter Simulation - Pile 2 - R3	39.45	77.22	5.47	10.98	8.56	2.42	90.23	2.93	83.03	6.33	1.02	90.38	3.60	1.35	4.95
E. dunnii - Winter Simulation - Pile 3 - R1	41.87	25.71	8.95	NR	NR	NR	NR	4.70	77.24	5.18	0.55	83.08	10.27	1.76	12.03
E. dunnii - Winter Simulation - Pile 3 - R2	42.47	27.63	9.02	NR	NR	NR	NR	4.69	77.14	5.38	0.64	83.27	10.47	1.80	12.27
E. dunnii - Winter Simulation - Pile 3 - R3	45.28	26.67	8.98	NR	NR	NR	NR	4.69	77.05	5.24	0.50	82.89	10.26	1.76	12.02

Table B2: Pulping data for simulated and control *E. nitens* chip piles

Sample ID	SPY	Viscosity	K - number	S10	S18	S10 - S18	Alpha cellulose	Copper number	Glucose (%)	Xylose (%)	Mannose (%)	Sum (%)	Klason Lignin (%)	Acid soluble lignin (%)	Total lignin (%)
E. nitens - Sappi Time zero pulping - R1	50.56	137.66	5.22	10.13	8.03	2.10	90.92	2.51	80.51	6.16	0.89	87.68	2.50	1.93	4.43
E. nitens - Sappi Time zero pulping - R2	51.10	121.55	4.76	10.08	8.08	2.00	90.92	2.47	80.76	6.18	0.83	87.89	2.92	1.71	4.63
E. nitens - Sappi Time zero pulping - R3	50.68	135.95	5.15	9.92	7.74	2.18	91.17	2.43	81.29	5.89	0.82	88.11	2.77	1.95	4.72
E. nitens - Control/Sappi - R1	48.95	52.76	4.65	10.74	7.62	3.12	90.82	2.67	85.15	5.37	0.65	91.17	3.15	1.63	4.78
E. nitens - Control/Sappi - R2	48.66	52.96	4.72	10.63	7.75	2.89	90.81	2.70	85.84	5.28	0.64	91.77	2.71	1.49	4.20
E. nitens - Control/Sappi - R3	48.88	53.89	4.72	10.74	7.56	3.18	90.85	2.68	86.06	5.27	0.81	92.14	3.49	1.62	5.11
E. nitens - Control/UKZN - R1	52.31	46.33	4.69	10.48	7.23	3.25	91.14	2.95	91.87	5.22	0.76	97.84	2.65	1.73	4.38
E. nitens - Control/UKZN - R2	52.31	52.16	4.65	10.37	7.18	3.19	91.23	2.93	88.61	5.19	0.68	94.48	2.56	1.50	4.06
E. nitens - Control/UKZN - R3	52.59	48.12	4.69	10.32	7.14	3.19	91.27	2.95	91.87	4.76	0.89	97.52	1.59	1.76	3.35
E. nitens - Summer Simulation - Pile 1 - R1	46.39	132.52	5.08	10.03	7.47	2.55	91.25	2.72	87.27	4.27	0.97	92.61	2.81	1.95	4.75
E. nitens - Summer Simulation - Pile 1 - R2	45.40	90.98	4.76	9.88	7.35	2.53	91.39	2.67	86.48	4.05	0.94	91.57	2.59	1.80	4.39
E. nitens - Summer Simulation - Pile 1 - R3	48.83	92.83	5.22	10.07	7.51	2.56	91.21	2.71	85.34	4.06	1.01	90.52	3.27	1.74	5.01
E. nitens - Summer Simulation - Pile 2 - R1	53.69	99.48	5.33	9.76	7.60	2.15	91.32	2.58	85.95	5.27	1.01	92.33	3.15	1.82	4.97
E. nitens - Summer Simulation - Pile 2 - R2	51.24	109.76	5.43	9.78	7.52	2.27	91.35	2.60	86.32	5.11	0.91	92.46	2.98	1.81	4.79
E. nitens - Summer Simulation - Pile 2 - R3	50.45	102.91	5.36	9.75	7.48	2.27	91.38	2.59	87.10	5.22	0.92	93.35	2.78	1.79	4.57
E. nitens - Summer Simulation - Pile 3 - R1	49.54	104.48	4.62	9.84	7.52	2.31	91.32	2.63	88.08	5.14	0.89	94.21	2.63	1.62	4.25
E. nitens - Summer Simulation - Pile 3 - R2	50.94	127.11	4.69	9.98	7.49	2.49	91.27	2.63	89.73	5.22	0.93	95.99	2.67	1.79	4.46
E. nitens - Summer Simulation - Pile 3 - R3	52.61	96.26	4.54	10.10	7.39	2.71	91.26	2.63	88.72	4.85	0.73	94.40	2.73	1.41	4.14
E. nitens - Winter Simulation - Pile 1 - R1	57.13	101.61	6.32	10.31	8.48	1.83	90.60	2.83	89.57	6.15	0.75	96.47	3.48	1.92	5.40
E. nitens - Winter Simulation - Pile 1 - R2	57.02	105.52	6.39	10.30	8.43	1.88	90.63	2.85	86.57	5.86	0.79	93.21	3.53	1.96	5.50
E. nitens - Winter Simulation - Pile 1 - R3	57.16	100.95	6.25	10.43	8.46	1.97	90.56	2.82	88.24	5.01	0.94	94.19	3.42	1.95	5.37
E. nitens - Winter Simulation - Pile 2 - R1	53.15	105.65	5.36	9.75	7.54	2.21	91.35	2.76	87.73	5.94	0.56	94.22	2.85	1.80	4.64
E. nitens - Winter Simulation - Pile 2 - R2	53	107.71	5.57	9.70	7.57	2.12	91.36	2.76	85.06	5.72	0.57	91.35	3.17	1.96	5.13

E. nitens - Winter Simulation - Pile 2 - R3	53.15	106.52	5.86	9.86	7.52	2.34	91.31	2.75	88.29	4.73	0.99	94.01	2.76	1.87	4.63
E. nitens - Winter Simulation - Pile 3 - R1	48.79	104.76	4.58	9.48	7.45	2.04	91.53	2.67	88.18	5.63	0.59	94.40	2.73	1.81	4.54
E. nitens - Winter Simulation - Pile 3 - R2	50	94.68	4.62	9.42	7.38	2.04	91.60	2.70	86.63	5.16	0.59	92.38	2.83	1.79	4.62
E. nitens - Winter Simulation - Pile 3 - R3	48.18	96.32	4.69	9.46	7.28	2.18	91.63	2.70	88.58	4.67	0.84	94.09	2.70	1.55	4.25

Table B3: Pulping data for simulated and control combination wood chip piles

Sample ID	SPY	Viscosity	K - number	S10	S18	S10 - S18	Alpha cellulose	Copper number	Glucose (%)	Xylose (%)	Mannose (%)	Sum (%)	Klason Lignin (%)	Acid soluble lignin (%)	Total lignin (%)
Combination - Summer Pile 1 - R1	49.03	82.75	4.72	10.48	8.34	2.14	90.59	2.98	84.30	5.24	0.81	90.46	3.37	1.78	5.15
Combination - Summer Pile 1 - R2	47.33	17.35	4.08	10.96	8.50	2.46	90.27	3.02	84.55	5.07	0.79	90.52	3.59	1.76	5.36
Combination - Summer Pile 1 - R3	48.48	82.95	4.86	10.48	8.27	2.21	90.62	2.99	85.22	5.22	0.82	91.36	3.48	1.84	5.32
Combination - Summer Pile 2 - R1	55.31	48.54	4.65	10.25	7.56	2.70	91.10	2.71	84.31	5.44	0.94	90.79	2.95	1.84	4.79
Combination - Summer Pile 2 - R2	47.89	59.37	4.54	10.33	7.60	2.72	91.04	2.72	84.59	5.38	0.89	90.97	2.89	1.81	4.70
Combination - Summer Pile 2 - R3	48.90	59.37	4.76	10.39	7.56	2.83	91.02	2.67	85.46	5.22	0.96	91.75	2.76	1.82	4.58
Combination - Summer Pile 3 - R1	52.89	146.58	4.83	11.10	8.21	2.89	90.35	2.69	85.57	5.51	0.83	92.02	3.35	1.88	5.24
Combination - Summer Pile 3 - R2	47.74	84.87	4.72	11.14	8.30	2.84	90.28	2.67	83.83	5.37	0.81	90.10	3.32	1.87	5.19
Combination - Summer Pile 3 - R3	47.96	69.79	4.90	11.34	8.31	3.03	90.18	2.68	85.28	5.53	0.78	91.69	3.42	1.87	5.30
Combination - Winter Pile 1 - R1	44.84	70.34	3.76	10.21	7.57	2.63	91.11	2.95	83.07	5.04	1.01	89.23	3.02	2.01	5.03
Combination - Winter Pile 1 - R2	46.67	68.01	3.80	10.13	7.57	2.56	91.15	2.95	82.08	4.85	1.05	88.09	2.92	1.83	4.75
Combination - Winter Pile 1 - R3	47.71	60.19	3.94	10.18	7.64	2.54	91.09	2.94	84.82	5.28	1.12	91.32	3.33	1.83	5.15
Combination - Winter Pile 2 - R1	46.02	74.73	3.98	10.62	7.52	3.09	90.93	3.01	84.03	5.05	1.07	90.26	3.08	1.83	4.90
Combination - Winter Pile 2 - R2	47.19	90.43	4.62	10.33	7.43	2.90	91.12	2.99	83.70	4.98	1.06	89.84	3.33	1.84	5.17
Combination - Winter Pile 2 - R3	45.81	66.30	4.26	9.92	6.85	3.06	91.61	3.10	84.46	4.42	0.90	89.90	3.09	1.54	4.63
Combination - Winter Pile 3 - R1	46.70	54.23	4.30	10.17	7.51	2.66	91.16	2.84	83.46	4.46	0.95	88.98	2.84	1.83	4.66
Combination - Winter Pile 3 - R2	48.56	53.54	4.40	10.41	7.87	2.54	90.86	2.88	85.60	4.44	1.04	91.20	2.89	1.74	4.63
Combination - Winter Pile 3 - R3	48.73	55.05	4.37	10.35	7.98	2.37	90.83	2.80	84.33	4.28	0.87	89.59	2.91	1.71	4.62