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Decay Fungi from New Zealand Leaky Buildings: Isolation, Identification and Preservative Resistance

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Abstract

Leaky buildings are those that show elevated moisture contents of the framing timber, which can subsequently lead to the establishment of fungal and bacterial decay. Prior to this study, the causative agents of the decay in these leaky buildings were unknown, though it was suspected to be one or more species of decay fungi. Therefore, the overall goal of this multi-disciplinary PhD thesis research was to determine the causative agents of decay in leaky buildings of New Zealand in an effort to develop solutions for both their remediation and future prevention.

Use of molecular biology methodology and classical mycological techniques based on morphology enabled identification of decay fungi from framing timber and air samples of leaky New Zealand buildings and provided insight into relative importance based on isolation frequency. In most cases, fungi colonising *Pinus radiata* D. Don were isolated to produce pure cultures. Mycelia from these cultures on agar media were collected to extract DNA. To identify the fungi to the species level, polymerase chain reaction (PCR) with fungal specific DNA primer pairs were performed followed by DNA sequencing of the internal transcribed spacer (ITS) region. Identification was by BLAST (Basic Local Alignment Search Tool) search on sequences in known GenBanks. In total, 421 samples from leaky buildings were processed, predominately untreated *P. radiata* decayed framing timber and also fibre cement boards and building paper. From these, sixty-eight fungal identifications were made. The only taxa that were isolated with significant frequency were identified as 4 basidiomycete species, as follows, along with the number of times they were isolated from the 421 samples:

➢ *Gloeophyllum sepiarium* (Wulf.: Fr.) Karst.	13x
➢ *Oligoporus placenta* (Fries 1865) Gilb. In Ryv.1985	11x
➢ *Antrodia sinuosa* (Fr.) Karst.	8x
➢ *Gloeophyllum trabeum* (Fr.) Murr.	4x

Although these species were identified repeatedly, in total they represent less than 10% of the total samples and, therefore, it is concluded that the leaky building decay samples represent high fungal biodiversity.

An aerial spore study of internal air, wall cavity air and exterior air of leaky buildings was carried out using a Merck MAS-100 instrument which collects spores directly onto selective media plates. Viable fungal aerial spores were detected at every sampling location tested at the leaky buildings, by the criteria of culturing, with a highest mean of 3714 colony-forming units (CFU) per cubic metre found in the cavities of water-damaged walls. This aerial spore study in conjunction with isolation from decayed wood samples from the same leaky buildings enabled identification of *G. sepiarium* and *A. sinuosa* at the same test site. The use of carboxymethylcellulose medium further demonstrated the presence of potential cellulose-degrading fungi within and around the location. Overall, the combination of direct sampling of timber and air sampling proved useful for detection of fungal species variability at a multi-unit building.

Four decay fungi isolated from New Zealand leaky buildings and two standard control decay fungi (*Coniophora puteana* and *Serpula lacrymans*) were submitted to laboratory wood block testing to determine the effectiveness of currently used wood framing preservatives under laboratory conditions before and after a standard leaching regime. *P. radiata* blocks were treated with water based boron & copper azole and solvent based IPBC & propiconazole plus tebuconazole (1:1) preservatives and exposed to the basidiomycetes for 12 weeks. Mass loss for the fungal decay-infected samples was recorded of up to 55% for preservative-treated samples, up to 62% mass loss for leached samples and up to 58% mass loss for un-preservative treated samples. Additionally, well defined dosage responses and approximate toxic thresholds were obtained for all preservatives tested. Results suggested that the minimum IPBC retention specified by Hazard Class 1.2 of NZS3640:2003 (0.025% m/m) is on the low side, and demonstrated after the 2 week leaching regime complete loss of efficacy of boron at 0.4% m/m boric acid equivalent (BAE).

This PhD research gave a first overview of fungi occurring in New Zealand leaky buildings, and it demonstrated the following key aspects of wood preservation:

1. The isolated test fungus *Antrodia sinuosa* was more difficult to control with propiconazole plus tebuconazole at retention 0.007% m/m than the known tolerant fungus *Oligoporus placenta*;
2. Boron at Hazard Class 1.2 retention of 0.4% m/m BAE was not toxic to *Oligoporus placenta*;
3. *Serpula lacrymans* exhibited tolerance to the highest retention of 0.06 %m/m tebuconazole plus propiconazole; and
4. *Gloeophyllum* species appeared susceptible to all wood preservatives.

In order to correlate fungal colonisation and wood decay, colonised wood blocks were studied using light microscopy (LM) and field- emission scanning electron microscopy (FE-SEM). Microscopic observations of *P. radiata* wood blocks following a standard wood decay test of twelve weeks of fungal colonisation by *Serpula lacrymans*, *Antrodia sinuosa*, *Oligoporus placenta* and *Gloeophyllum sepiarium* revealed that the two microscopic techniques employed were complementary by allowing features such as pit membranes, chlamydospores or S3/S2 compound middle lamella interface to be photographed in greater detail, allowing for more precise analyses and interpretation of key findings, as follows:

1. Brown rot fungi directly target their apical growth towards degraded pit apetures;
2. Reliance on light microscopy and observed birefringence as a tool to record changes in cell wall crystallinity associated with brown rot decay alone could be misleading;
3. Presence of fine ($\leq 1 \mu m$) to wide (≥ 3.5 µm) bore-hole and hyphal size ranges, and nearly unchanged cell wall thickness of all wood/test fungal combinations, confirmed active decay at moderate to late stages;
4. Some ray parenchyma cells for *Antrodia sinuosa*, *Oligoporus placenta* and *Gloeophyllum sepiarium* colonised blocks were intact throughout late stages of decay, outlining that they were not preferentially degraded early in the brown rot decay process, and

5. Presence of bore-holes, clamp and medallion clamp formation and resting spores (chlamydospores and arthrospores) are fungal specific, can aid in their differentiation and identification, and should be recorded during wood decay studies, as especially resting spores are an important factor when planning remediation strategies.

In summary, this PhD thesis research provided the first comprehensive investigation into the biodiversity of fungi from leaky New Zealand buildings, identified the dominant species and presented details about their micromorphology and their decay patterns. It also demonstrated substantial differences in efficacy of preservative formulations currently (December 2008) approved for framing treatments in New Zealand and possible deficiencies where framing may be subjected to severe leaching. This study also provided the first comparative analyses of viable fungal aerial spores between leaky wall cavities and the surrounding air environment. Subsequently, this research added to the knowledge of the decay fungal species diversity in and around New Zealand leaky buildings, outlined their capabilities to degrade treated and un-treated *P. radiata* framing timber and illustrated the efficacy of New Zealand approved wood preservatives for their potential as remedial treatment and future prevention.

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Dedicated
to
my wife

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Chapter 5

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Nomenclature of fungi

This table lists the full name of the fungus, the naming authority and date.

Species

Synonym(s)

Antrodia sinuosa (Fries) P. Karsten (1881)

Polyporus sinuosus Fr. (1821)
Trametes sinuosa (Fr.) Cooke & Quél.
Poria sinuosa (Fr.) Cooke (1886)
Polystictus sinuosis (Fr.) Lloyd (1917)
Polyporus sinuosus Fr. (1821)
Physisporus sinuosus (Fr.) Gillet (1878)
Irpex lacteus subsp. sinuosus (Fr.) Bourdot & Galzin (1925)
Coriolus sinuosus (Fr.) Bondartsev & Singer (1941)
Coriolellus sinuosus (Fr.) A.K. Sarkar (1959)
Spongiporus sinuosus (Fr.) Aoshima (1967)

Gloeophyllum sepiarium (Wulfen) P. Karsten (1882)

Daedalea sepiaria (Wulfen) P. Gärtner, B. Meyer & Scherb. (1802)
Lenzites sepiaria (Wulfen) Fr. (1838)
Agaricus sepiarius Wulfen (1786)
Merulius sepiarius (Wulfen) Schrank (1789)

Gloeophyllum trabeum (Persoon) Murrill (1908)

Agaricus trabeus Pers. (1801)
Daedalea trabea (Pers.) Fr. (1821)
Daedalea quercina subsp. trabea (Pers.) Pers. (1828)
Lenzites trabea (Pers.) Fr. (1838)
Trametes trabea (Pers.) Bres. (1897)
Cellularia trabea (Pers.) Kuntze (1898)
Coriolopsis trabea (Pers.) Bondartsev & Singer (1941)
Agaricus trabeus Pers. (1801)
Lenzites trabeus (Pers.) Bres. (1897)
Phaeocoriolellus trabeus (Pers.) Kotl. & Pouzar (1957)

Oligoporus placenta (Fries) Gilbertson & Ryvarden (1985)

Polyporus placenta Fr. (1861)
Physisporus placenta (Fr.) P. Karst. (1882)
Poria placenta (Fr.) Cooke (1886)
Leptoporus placenta (Fr.) Pat. (1900)
Ceriporiopsis placenta (Fr.) Domanski (1963)
Tyromyces placenta (Fr.) Ryvarden (1973)
Postia placenta (Fr.) M.J. Larsen & Lombard (1986)
Rhodonia placenta (Fr.) Niemelä (2005)

Alternaria Nees (1816)

Antrodia carbonica (Overholts) Ryvarden & Gilbertson (1984)

Antrodia vaillantii (DeCandolle) Ryvarden (1973)

Antrodia xantha (Fries) Ryvarden (1973)

***Armillaria* sp.** (Fries) Staude (1857)

Armillaria hinnulea Kile & Watling 1983

Armillaria mellea (Vahl) P. Kummer (1871)

Aspergillus nidulans (Eidam) G. Winter (1884)

Botrytis P.Micheli ex Persoon (1794)

***Cadophora* sp.** Lagerberg & Melin (1927)

Ceriporia lacerata N. Maekawa, Suhara & R. Kondo (2003)

***Ceriporiopsis* sp.** Domański (1963)

Cladosporium Link (1816)

Coniophora puteana (Schumacher) P. Karsten (1868)

Coprinellus micaceus (Bulliard) Vilgalys, Hopple & Jacq. Johnson (2001)

Coprinus domesticus (Bolton) Gray (1821)

***Epicoccum* sp.** Link (1815)

Fomes fomentarius (Linnaeus) Fries (1849)

Fomitopsis palustris (Berkeley & M.A. Curtis) Gilbertson & Ryvarden (1985)

***Fusarium* sp.** Link (1809)

***Ganoderma* sp.** P.Karsten (1881)

Geotrichum Link (1809)

***Gliocladium* sp.** Corda (1840)

Heterobasidion annosum (Fries) Brefeld (1889)

Inonotus hispidus (Bulliard) P. Karsten (1879)

Lentinus squamosus (Schaeffer) Quélet (1888)

***Mortierellales* sp.** Cavalier-Smith (1998)

Mucor Fresen. (1850)

Neurospora crassa Shear & B.O. Dodge (1927)

***Paecilomyces* sp.** Bainier (1907)

***Penicillium* sp.** Link (1809)

Pestalotiopsis neglecta (Thümen) Steyaert (1953)

Phanerochaete chrysosporium Burdsall (1974)

***Phanerochaete* sp.** P.Karsten (1889)

Phellinus contiguus (Persoon) Patouillard (1900)

Phellinus pini (Brotero) A. Ames (1913)

Phlebia uda (Fries) Nakasone (1997)

Phlebiopsis gigantea (Fries) Jülich (1978)

Pleurotus subpalmatus (Fries) Gillet (1876)

Polyporus arcularius (Batsch) Fries (1821)

Polyporus brumalis (Persoon) Fries (1818)

Pycnoporellus fulgens (Fries) Donk (1971)

Resinicium friabile Hjortstam & Melo (1997)

Rigidoporus laetus (Cooke) P.K. Buchanan & Ryvarden (1988)

Serpula himantioides (Fries) P. Karsten (1884)

Serpula lacrymans (Wulfen) P. Karsten (1884)

Sistotrema brinkmannii (Bresadola) J. Eriksson (1948)

***Streptomyces* sp.** Waksman & Henrici (1943)

Trametes versicolor (Linnaeus) Lloyd (1920)

Trichoderma citrinoviride Bissett (1984)

Ulocladium Preuss (1851)

Glossary of Terms / List of Abbreviations

Å	Ångström
A	Ascomycete
ACQ	Alkaline copper quaternary
AMA	Acidic malt extract agar
AWPA	American Wood Preservers Association
B	Basidiomycete
BAE	Boric acid equivalent
BLAST	Basic Local Alignment Search Tool
BMA	Basidiomycete semi-selective agar
bp	Base-pairs
BR	Brown rot
BRANZ	Building Research Association of New Zealand
BS	British Standard
C.I.	Colour Index
CCA	Copper chrome arsenate
CDH	Cellobiose dehydrogenase
CFU/m^3	Colony forming units per cubic metre
CMC	Carboxymethylcellulose
CTAB	Cetyltrimethylammonium bromide
CuAz	Copper azole
CuN	Copper naphthenate
DDAC	Didecyl dimethyl ammonium chloride
DDBJ	DNA Data Bank of Japan
DNA	Deoxyribonucleic acid
EB	Erosion bacteria
EC	Enzyme Commission number
ECMM	Extracellular mucilaginous material
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EMC	Equilibrium moisture content
EN	European Standard

E-value	Expect-value
FE	Field-emission
FE-SEM	Field-emission scanning electron microscopy
FSP	Fibre saturation point
GenBank	Collection of publicly available nucleotide sequences, produced by the National Centre for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database collaboration (INSDC)
H	Hazard class
IGS	Intergenic spacer
IPBC	3-Iodo-2-propynyl-butyl-carbamate
ITS	Internal transcribed spacer
kGy	Kilogray
kPa	Kilopascal
LiP	Lignin peroxidase
LM	Light microscopy
LOSPs	Light organic solvent preservatives
MA	Malt agar
MC	Moisture content
ML	Mass loss
ML	Middle lamella
MnP	Manganese-dependent peroxidase
MOE	Modulus of elasticity
MOR	Modulus of rupture
NCBI	American GenBank
nm	Nanometer
NMR	Nuclear magnetic resonance
NZ	New Zealand
NZBC	New Zealand Building Code
NZS	New Zealand Standards
P	Primary cell wall
PCR	Polymerase Chain Reaction method, which enables exponential amplification of target DNA
RA	Relative Enzyme Activity Index

RAPD-PCR	Randomly amplified polymorphic DNA by the polymerase chain reaction
rDNA	Region of chromosomal DNA that codes for ribosomal RNA
RH	Relative air humidity
S1, S2, S3	Wood cell wall layers
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SR	Soft rot
SYP	Southern yellow pine species
T	Temperature
Taq	Thermostable DNA polymerase, named after thermophilic bacterium *Thermus aquaticus*
TB	Tunneling bacteria
TBTN	Bis-(tri-n-butyltin) naphthenate
TBTO	Bis-(tri-n-butyltin) oxide
T-RFLP	Terminal Restricted Fragment Length Polymorphism
UV	Ultraviolet light
W	Warty wood cell wall layer
WC	White collar complex
WHRS	New Zealand Government´s Weathertight Homes Resolution Service
WR	White rot
YM	Malt Yeast Extract Agar
Z	Zygomycete
%m/m (retention)	% mass per unit mass
µm	Micrometer
µW m^{-2}	Microwatts per square metre

1. Introduction, Literature Review and Objectives

1.1 Introduction

For the past ten years, leaky buildings in New Zealand have been of great concern. These buildings can be defined as lacking the ability to adequately withstand the weather conditions in New Zealand thereby allowing water to penetrate the building envelope or cladding system and then hold the water in the wall cavity, where it may remain for some time. The New Zealand Government's Weathertight Homes Resolution Service (WHRS), which manages claims from homeowners of leaky buildings, has processed 5,800 cases since its establishment in 2002. There are also a large unknown number of private claims and presumably more leaky houses that are not linked to claims. The framing timber used predominately in these houses was *Pinus radiata* D. Don, a non-durable native gymnosperm of California that is extensively cultivated in New Zealand with an 80% market share in light timber framing (other framing materials include *Pseudotsuga menziesii* (Douglas-fir) and Steel) (BRANZ research data, New Zealand Herald, 2007).

Framing timber that is not adequately protected from changeable weather conditions, for example being poorly protected by cladding, might be exposed to prolonged moisture situations and is subsequently susceptible to decay. This PhD thesis research sought to isolate and identify causative agents of fungal decay in leaky buildings and to increase understanding of their ecology, with the research including the following:

- Use of the latest molecular techniques for decay fungal identification.
- Study of degradation of variously treated framing using common timber preservatives and fungal isolates from leaky buildings.
- Collection of viable, airborne fungal spores at leaky building sites to describe the environmental situation surrounding the framing timber in service.
- Characterisation of micromorphological changes caused by decay fungi in *Pinus radiata*.

1.2 The leaky building crisis and its current (2008) situation

The exotic softwood *Pinus radiata* (Radiata or Monterey Pine) has been used as a building material in New Zealand homes since the first plantation logs became available in the 1950s. *P. radiata* was a substitute for diminishing supplies of native softwoods like *Dacrydium cupressum* (Lamp.)(rimu). Initially, *P. radiata* was used untreated but it was soon realised that it was susceptible to borer attack and was therefore treated with boron to protect it. Timber used in outside situations like foundation poles in ground contact were treated with Copper Chrome Arsenate (CCA) to secure longer service-life. During the 1980s, more and more construction timber was artificially dried in kilns, and it was found that high temperature drying (above 100°C) provided some resistance to insects. No consideration, however, was given to the study of fungal degradation of kiln-dried timber. A decision made by the New Zealand Standards Committee in 1995 allowed the use of untreated, high temperature kiln dried *P. radiata* in place of boron treated timber under conditions where it could be kept dry, with a moisture content (MC) not greater than 18% at all times, and clear of the ground (NZS 3602: 1995). The decision was based on BRANZ and Forest Research Institute research data; Grant Hardie of BRANZ concluded in a **Build Magazine** article in 1997: "there is no scientific basis for timber treatments to be made a mandatory requirement for framing of Radiata pine. This means that to treat or not to treat is a choice for the building owner alone" (Hardie 1997).

The co-factor that no one had considered at that time was the introduction of new construction materials (i.e. stucco as cladding material, wall insulation) and new fashions in building design (i.e. flush eaves design). A considerable number of houses were built using methods and products that were unable to withstand the sometimes harsh (and coastal) weather conditions of New Zealand, allowing moisture into the framing timber in the walls. Additionally, some cladding types didn't allow for drainage cavities (Section 1.2.2) with the result that water got entrapped within the walls, leading to a rise of the moisture content of the framing, which was subsequently prone to fungal decay.

In 2002, the Department of Building and Housing appointed a Weathertightness Overview Group to investigate the problem, who then issued a report known as the Hunn report (Hunn *et al.* 2002), which led to the establishment of the main factors involved in leaky buildings (as discussed in Section 1.2.3) and to the formation of the governmental WHRS to assist affected home owners in their disputes.

Since early 2004, amendments have been made to the New Zealand Standard 3602: 2003 Timber and wood-based products for use in buildings, and the New Zealand Building Code Clause B2: Durability (NZBC (2004)), requiring again the incorporation of treated timber for certain applications (Section 1.8.2).

1.2.1 Affected buildings

Decay in buildings is typically associated with failure of one or more of what have been referred to as the 4D's: deflection, drainage, drying and durability (Hazleden & Morris 1999; Section 1.2.2). Many houses constructed in New Zealand during the 1990s were deficient with respect to one or more of these parameters. Some of these deficiencies were inherent within the design itself and others were caused by poor workmanship and lack of attention to detail. Of particular concern were 'Mediterranean style houses' where eaves were unfashionable and flashings were inadequate or were missing; monolithic claddings with little drainage and drying ability became popular, and untreated timber was used instead of traditional boron treated framing (Hunn 2002).

A study of 287 pre-purchase inspection reports during 1996-99 of buildings constructed since 1970 revealed that at least two defects were found at every house inspected, either affecting the fabric of the cladding or cladding junctions (Murphy 2001), and about 5% of all houses were significantly defective. It was suggested that there were multiple reasons for the increase in cladding defects including rapid introduction of stucco and flush-jointed, rigid sheet materials as claddings, lack of trade skills, poor sealant use and a preference for designs with flush eaves. Thirty percent of both the stucco and textured fibre-cement defect cases were recorded as having moisture elevation issues. Moisture accumulation within the walls can ultimately lead to structural failure of wood and other building materials, which contain natural fibres, due to fungal and bacterial decay as shown in Figure 1.1, which is a photograph of decayed framing from a recent (2008) survey at a multi-unit building complex in Auckland.

Figure 1.1 *Gloeophyllum sepiarium* fruiting body (arrow) on framing stud and associated white mycelium. Framing timber used was *P. radiata*.

1.2.2 Weathertightness

The term weathertightness is used frequently to discuss the envelope design of buildings and to determine if it might allow water to penetrate into the structure. As an adjective, in terms of a building, weathertight simply means "sealed against rain and wind". However, weathertightness is not necessarily "waterproofing" of a building, which is more the combination of good design and appropriate building material usage and installation. This is best expressed through the mentioned building model incorporating the 4 D's: deflection (1), drainage (2), drying (3) and durability (4) (Hazleden & Morris 1999) as depicted in Figure 1.2., which achieves the following to protect the buildings:

- **Deflection** (1) of driving rain through eaves or flashings to keep moisture away from sensitive areas;
- **Drainage** (2) of moisture that entered into the construction by creating pathways for the water to drain down and out of the wall;
- **Drying** (3) of moisture in the walls through provision of ventilation gaps;
- **Durability** (4) of materials used to construct walls and roofs ensure an adequate level of life expectancy for the situation they will be used in.

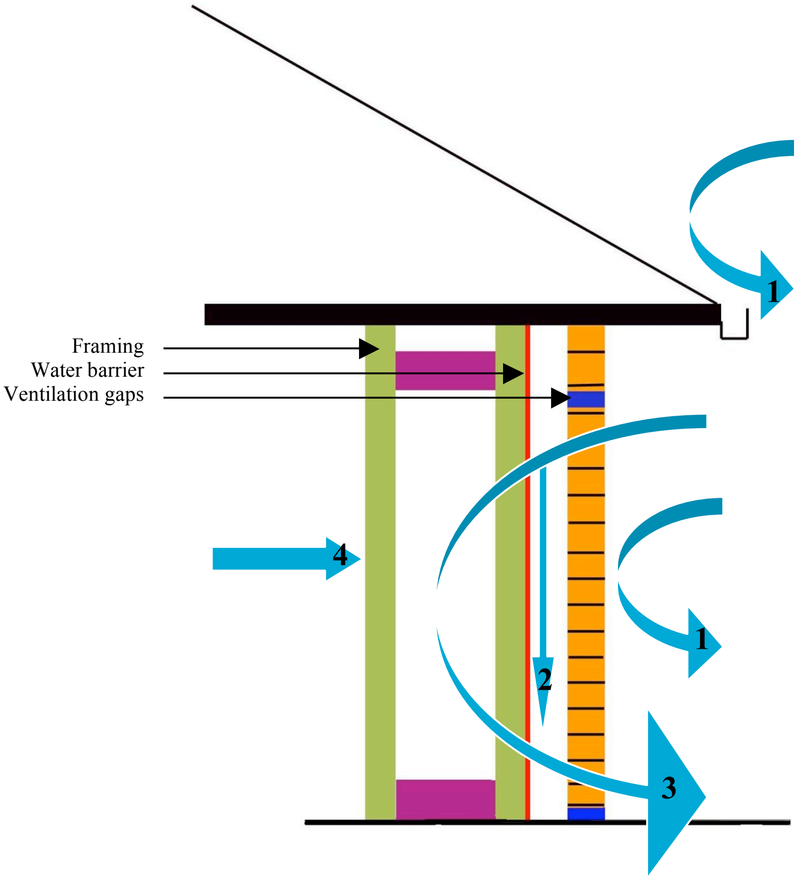


Figure 1.2 Drafted diagram showing the 4 D's: deflection (1), drainage (2), drying (3) and durability (4).

1.2.3 Common leakage problems

Designing and building a house is a complex procedure, requiring the expertise of many different trades and professionals as well as adherence to government standards, regulations and guidelines for building procedures and products. Only if these multiple influencing factors interact in harmony can the consumer expect a house to be built to standard and achieve the estimated life expectancy of all building components. However, as in the 1990s in New Zealand, things went wrong and some factors did not conform to the collectivity any more. The result of this, or in other words the end product house, was often defective in one or more aspects. Probably the most serious threat to the house was defects to the envelope protecting the inner structural building components. Once construction timber was repeatedly exposed to the effects of weather and moisture accumulation the building became prone to natural degradation of the timber, with the possible end result of an uninhabitable house. Common leakage problems were identified in 2002 with the 'Hunn Report' and by the New Zealand Department of Building and Housing, assessing leaky house data of the WHRS claims for the year 2007. Contributing factors were multiple and often involved the design of the building and the installation, sometimes even inappropriate use of the construction materials (Groufsky 2008). According to Groufsky (2008), today's "most common causes of building failures resulting in leaks are cap flashings, window flashings, cracks in external cladding and cladding to the ground clearance." Many of the factors identified can be summarised as follows: (not in order of importance)

- **'Mediterranean' style buildings** in combination with the use of monolithic and directly fixed cladding systems not allowing for drainage or ventilation;
- **Complex design features** like two or more storeys, dormer windows/skylights, flat roofs with narrow or no eaves, solid balustrades and parapets, complex roof design, balconies that jut out from the walls, penetrations through the claddings;
- **Non-durable materials** i.e. untreated timber;
- **Flashings** inadequately or poorly constructed around doors and windows;

- **Lack of technical knowledge and skills** when houses are designed, detailed and built.

(Summary of Department of Building and Housing New Zealand data, available on www.consumerbuild.org.nz)

Concerning periodic moisture build-up, water might reach the framing timber not only through defects in the building envelope but can also be related to one or more of the following factors according to Ridout (2000) and Huckfeldt & Schmidt (2006a):

- **Condensation**, i.e. on window glass and frame, water vapour emissions through living activities such as cooking, taking a shower or heating, non-insulated cold water pipes.
- **Faults at water conducting pipes**, i.e. inappropriate installation, blocked water drains, defects at water mixers.
- **Ground water**, i.e. lack of drainage, defective water barrier in the foundation, root systems of trees that penetrate the building envelope, floods.
- **Rainwater**, i.e. no maintenance of the building envelope and storm water pipes such as defects of roofs, gutters, cracked paint on weatherboards or defects of cladding materials, windows and doors.

In summary, the most important questions when examining construction timber and leaks are: a) if the critical moisture limits conductive or hindering decay were or were not present at a certain point in time, b) how moisture fluctuations and duration influenced the decay rate, and c) how deep the water was able to penetrate into the timber?

1.3 Wood decay fungi

In this section of the thesis, the discussion focuses on the appearance, structure and reproduction of wood decay basidiomycota (basidiomycetes), as fungi belonging to this group were found to be the dominant decay species in buildings in New Zealand within this study, and by others in European studies of leaky building (Schmidt 2006). The discussions concerning fungal descriptions and decay mechanisms were generally based on Rypáček (1966), Bech-Andersen (1995), Jennings & Lysek (1996), Huckfeldt & Schmidt (2006a) and Schmidt (2006), or as specifically referenced.

1.3.1 Appearance, structure and reproduction principles in decay fungi

The occurrence of decay fungi in buildings constructed of timber is not uncommon and is usually associated with moisture related problems as outlined in Section 1.2. The appearance of decay fungi is basically the same as observed on felled logs in the forest but is much more difficult to detect in enclosed framing timber. Early decay, sometimes referred to as incipient decay, can easily be overlooked as thin hyphae might be hidden between the inner/outer cladding and the framing members. However, once the framing is exposed, the decay features are the same and can be divided into three categories:

- Thin walled substrate hyphae which cause depolymerisation of the wood cells and provide the fungus with the necessary nutrients and energy (Section 1.5.6).
- Thick walled surface mycelium with narrow luminae that often produce string-like mycelial cords (strands), sometimes-called rhizomorphs, with the role to locate other suitable substrates.
- Fruiting bodies that have the role of reproduction through the formation of spores.

1.3.1.1 Structure of the fungal hyphae

Fungi are eukaryotic and lack the ability to photosynthesise, meaning they are achlorophyllous and rely on an external source of carbon for their growth and extension. As a result, fungi produce hypha (pl. hyphae), a tool fungi use to achieve colonisation and degradation of carbon rich substrates like wood. Basically, a hypha is a long filamentuous cell of cylindrical or tube-like shape surrounded by a cell wall. This cell wall contains a plasma membrane (plasmalemma) and is in most fungi composed of chitin and ergosterol, the detection of the latter is sometimes used for fungal quantification, enclosing the cytoplasm. Unless the fungus is aneuploid, the cytoplasm contains either one nucleus (1n) or two nuclei (1n+1n) (see Section 1.3.1.2). Further, other organelles like the vacuolar system and its bounding membrane, the tonoplast, mitochondrion or Woronin bodies are contained in the protoplasm (Figure 1.3). Woronin bodies block the septal pore(s) if a hyphal compartment is physically ruptured, preventing the loss of contents in undamaged compartments; hence, that part can continue growth or spread of the fungus.

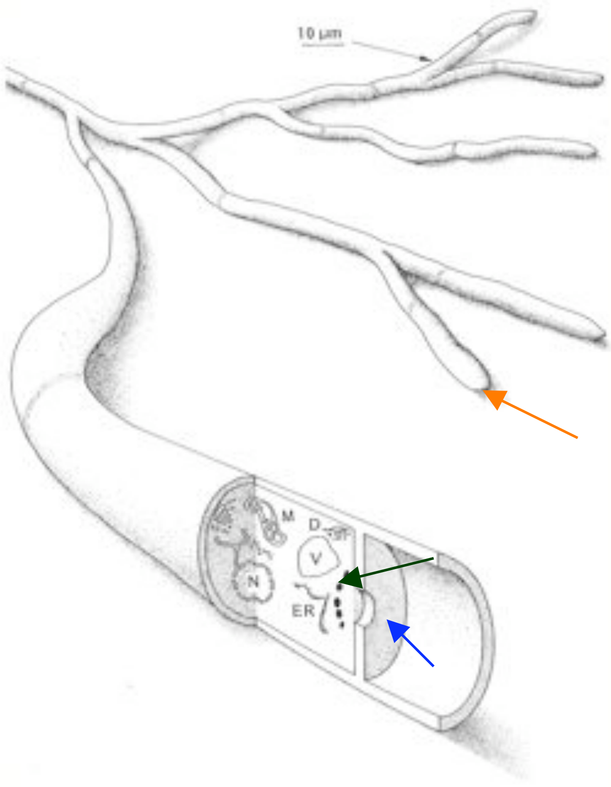


Figure 1.3 The hyphal branching system and apical growth (orange arrow). The sectioned part exposes the septum (blue arrow) and features in the protoplasm: (**N**) nucleus, (**ER**) endoplasmatic reticulum, (**D**) dictyosome, (**V**) vacuole, (**M**) mitochondrion, Woronin bodies (green arrow). Source: Jennings & Lysek (1996).

The fungal cells or hyphae produced by germination of a spore are called generative hyphae (sometimes vegetative hyphae), and in most wood-inhabiting basidiomycetes these single cells are divided by a semi-permeable membrane named septum (pl. septa) (Figure 1.3) that can have one pore as in *Ascomycetes* or two pores (dolipore septum) as in *Basidiomycetes*.

Within the fungal cell a positive osmotic (hydrostatic) pressure is created by the vacuolar system that generates the turgor, providing the cell with mechanical strength outward to not collapse, and inward for hyphal extension. For this, a very complex cell wall system is required (Robson 1999; in Schmidt 2006) and in basidiomycetes this is multilamellar. In general, the wall is made up of various polysaccharides, mainly ß-linked polymers of glucose and of chitin, a polymer composed of *N*-acetyl glucosamine units. As well, an important component of the cell wall is termed the slime layer or mucilage layer, composed of protein, lipid and carbohydrates or of crystalline to membranous and fibrillar structures. This layer "glues" the fungal hypha to the wood cell wall and possibly has some of the following functions in the decay process:

- Covering the S3 layer of the wood cell wall during decay process.
- Conditioning of the substrate for decay.
- Transport vector for low-molecular decay agents and enzymes to the wood.
- Transport vector for degradation products to the hypha.
- Microenvironment for H_2O_2 maintenance for lignin degradation.
- Storage of nutrients.
- Storage of copper or CCA from attack of impregnated wood.

(Excerpt of possible slime layer functions according to a review by Schmidt (2006))

Hyphal extension is by apical (tip) growth of the fungal cell (Figure 1.3). This small area of the hypha increases its wall by cylindrical insertion of precursors namely microfibrils, comprised of unbranched long-chain polymer chitin (in Eumycota) and some smaller molecular weight polymers interspersed between the fibrils. The hyphal tip is sometimes referred to as the dome and is the most sensitive part of the mycelium. It features different vesicles and membranous structures for cell wall synthesis and transport processes. Enzymes secreted from the tip for nutrient metabolism are one of the many processes.

1.3.1.2 Mycelium and cord formation (cell division)

Basidiomycetuous spores possess a halved chromosome number (1n) and are called haploid. Once landed on a suitable substrate (right temperature, moisture, as discussed in Section 1.5), the spores will germinate and produce very thin hyphae. After repeated divisions, these hyphae form a dense web called mycelium and is often referred to as primary mycelium, the monocaryont. These haploid hyphae can fuse with other haploid hyphae originating from the germination of another spore, and form together the secondary mycelium containing now two haploid nuclei (dicaryons) from two different parental genomes (1n+1n) and is called heterocaryont. This heterocaryont mycelium is the actual vegetative stage of a fungus where the two fused nuclei have not yet undergone the process of karyogamy. Particularly, in basidiomycetes, this dicaryotic stage is often prolonged through conjugated mitosis. Hyphal growth through cell division in many basidiomycetes, when in a dicaryotic phase, produce clamp connections. During this division, a special nuclear migration takes place resulting in a clamp connection, combining two daughter cells which both become dikaryotic again. These clamp connections may or may not be found often at every cell dividing septa or only sparsely. However, if detected, it is a first distinguishing feature for basidiomycete fungi. The act of nuclear migration and clamp formation is delineated in Figure 1.4.

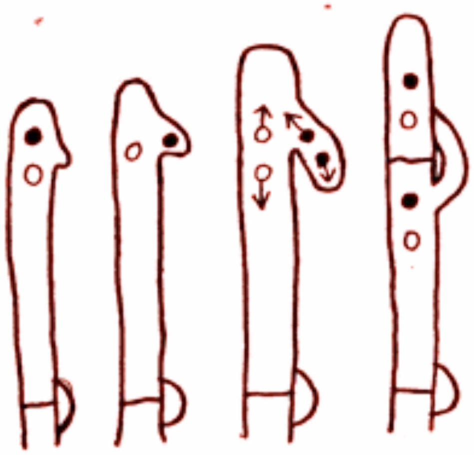


Figure 1.4 Delineation of clamp formation and cell division in dicaryotic basidiomycete hyphae. Redrawn from Rypáček (1966).

The two types of hyphae formed during the vegetative stage of the fungus are the substrate hyphae and the surface hyphae constructing the mycelium (Figure 1.5). Substrate hyphae are thin walled, rich in plasma and are responsible for the nutrition of the fungus. The surface hyphae are the same but often strands/cords or rhizomorphs are produced from the surface mycelium that are usually thick walled in nature and have narrow lumina. The literature is at variance about using the terms strands/cords or rhizomorphs. Rhizomorphs literally are root-like aggregations of hyphae that have a well-defined mycelial cord (apical meristem) and are frequently differentiated into a rind of small melanised cells (sheathing hyphae) surrounding a central core of elongated colourless cells (vessel hyphae). Strands (cords) formed in the mycelia of wood decay fungi consist of vegetative hyphae, thin fibre (skeletal) hyphae with thick cell walls for strengthening of the strands and broad vessel hyphae mainly without any septa. The main function of both rhizomorphs and strands are nutrient translocation through the elongated and wide vessel hyphae. According to Schmidt (2006), there are two characteristics that differentiate rhizomorphs and strands; the latter produces a not-so-well organized longitudinal mycelium that can develop behind the mycelial growth front and overgrow unsuitable substrates to reach nutrients at a distance (e.g. *Serpula lacrymans*). However, rhizomorphs produced by, for instance, *Armillaria* sp. are known to cover distances between trees (Jennings & Lysek 1996).

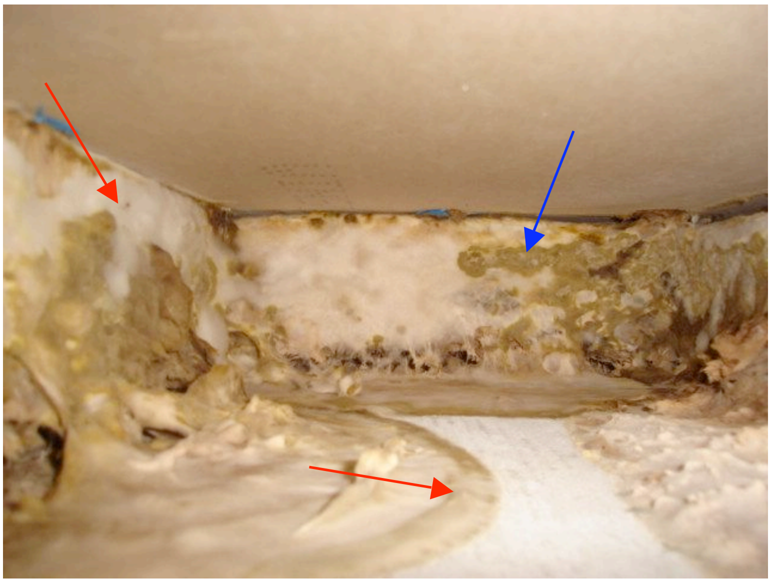


Figure 1.5 Advancement of mycelium (red arrows) in wall cavity and secondary mould growth (blue arrow). Source: Beagle Consultancy Ltd, Rotorua, NZ.

Most building decay fungi will produce strands (Figure 1.6), which are, together with fruiting bodies, the first distinguishing sign for fungal identification. In 1912, Falck differentiated between some building decay fungi by the means of strand diagnosis for species such as S. *lacrymans*, *Coniophora puteana* and *Antrodia vaillanti*. Today, there are updated identification keys for strand forming fungi available. (Huckfeldt & Schmidt (2006b), which are discussed more in Section 1.7.

Figure 1.6 Strand formation of a basidiomycete fungus on building paper (arrows).

1.3.1.3 Fruiting body formation

Fruiting body formation occurs if environmental factors change (temperature, air humidity, light, oxygen; as discussed in Section 1.5) and/or the substrate is nearly depleted of its nutrients. The fruiting body develops out of the dicaryotic secondary mycelium (s.M.), at the surface of the substrate and is sometimes referred to as tertiary mycelium or basidiocarp. As drawn in Figure 1.7, decay fungi develop fruiting bodies (F) (not limited to buildings as they occur mainly in the forest) and belong to the genus hymenomycetes, as they form a fertile layer called the hymenium (H). This layer varies in shape and form and can be used to differentiate between fungal species. For example, it can be porous or lamellate, producing warts or net-like arrangements. Within this hymenium, the formation of the basidium (pl. basidia) takes place, which later bears the haploid basidiospores. The mechanisms of how these spores are produced by the fungus are also illustrated in Figure 1.7. Within the young basidium (I), which contains the two parental nuclei, first karyogamy (II) occurs (mergence of the two nuclei), followed by meiosis (III) producing four (in some cases only two) haploid nuclei that subsequently migrate into the endogenous sterigmata (outgrowths) at the top of the basidium. Within each sterigmata tip sits one basidiospore (IV) waiting to be discharged to germinate again into monocaryons. How these spores and fruiting bodies can vary in size, shape and colour is discussed in Section 1.7, identification of wood decay fungi.

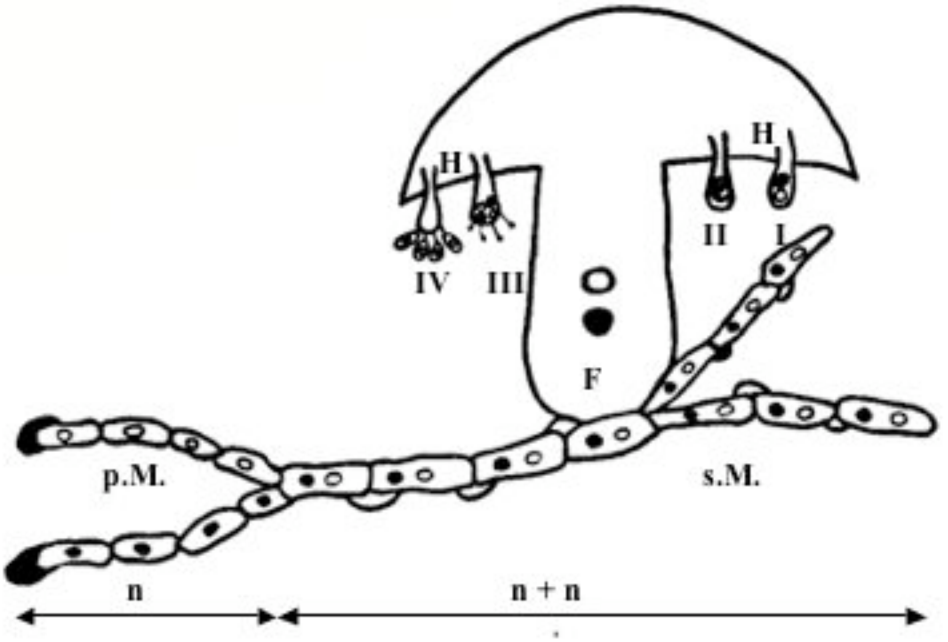


Figure 1.7 Mechanisms of fruiting body and spore formation in homobasidiomycetes. In parts redrawn from Rypáček (1966). (**n**) haploid nuclei, (**p.M.**) primary mycelium, (**s.M.**) secondary mycelium, (**F**) fruiting body in dicaryotic stage, (**H**) hymenium layer of fruiting body, (**I**) formation of basidia containing two nuclei, (**II**) karyogamy, (**III**) meiosis, (**IV**) formation of four haploid basidiospores located in sterigmata.

1.4 Growth establishment on wood

The basic pathway of fungal growth establishment on wood with particular consideration of *P. radiata* construction timber and its natural durability is discussed in this section.

1.4.1 Structure of Pinus radiata wood

In general, the basic axial cellular structure of wood consists of millions of parallel, thick walled and elongated cells, which form together the wood tissue. Softwoods like *P. radiata* have cells that are referred to as tracheids, whereas in hardwoods like *Quercus* sp. these cells are called fibres and vessels. Figure 1.8 shows an SEM picture of *P. radiata* wood in longitudinal, radial and transverse faces.

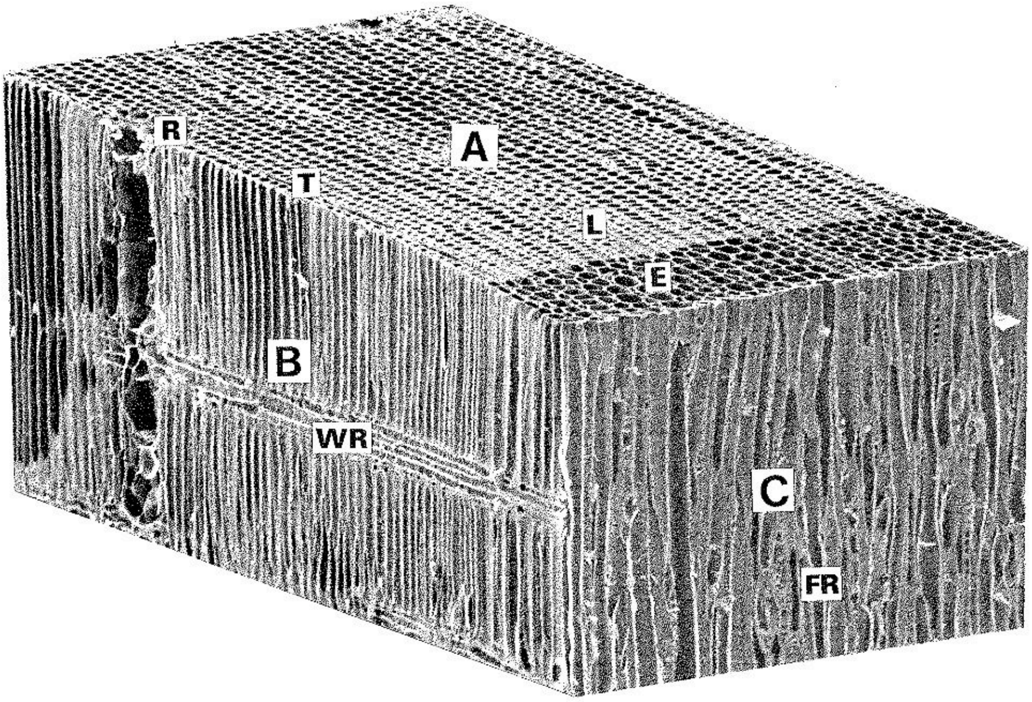


Figure 1.8 Scanning electron micrograph of *P.* radiata:
Face A: Transverse: T= cut ends of tracheids; L= latewood; E= earlywood; R= resin canal; Face B: Radial/longitudinal: WR= wood ray; Face C: Tangential/longitudinal: FR= fusiform ray; Source: Harris 1991.

The tracheids of *P. radiata* account for approximately 95% of the timber by volume. Radial and axial parenchyma cells and resin canals account for the other 5%. Further, various extractives might be stored in i.e. resin canals. These are often classified as crystallisation resistant resins, namely terpenes, lignans, stilbeans, flavonoids or other aromatics. Further there occur fats, waxes, fatty acids, alcohols, steroids and higher hydrocarbons.

In radial orientation of the wood structure, short and thin walled parenchyma cells form the ray tissues. These parenchyma cell walls have high water permeability and have many pits on all sides. These pits connect the wood cells with each other as can be seen in Figure 1.9.

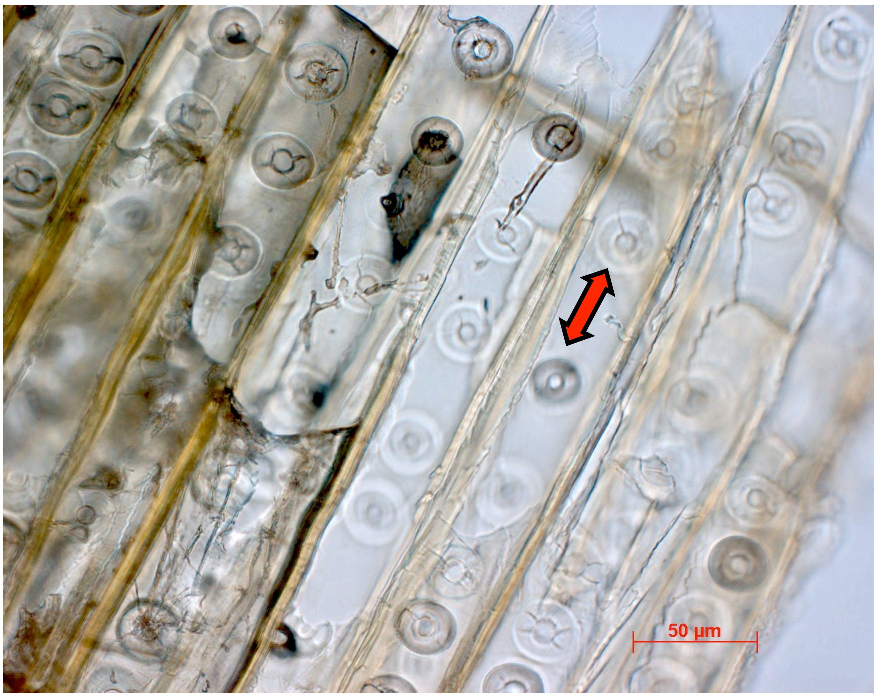


Figure 1.9 Microtome section of *P. radiata* tracheids detailing pit structure (red arrowheads).

Wood cell walls of tracheids or fibres consist of a very thin primary wall of microfibrils followed by the secondary cell wall comprised of three layers called S1, S2 and S3. The central region of the primary cell wall that separates neighbouring cells is called the middle lamella. The S2 layer, the thickest of all three, gives the cell wall most of its strength, as densely packed cellulose microfibrils follow a parallel orientation to the cell axis. Sandwiched between is the much thinner hemicellulose-rich S1, which is often encrusted with lignin and is connected to the middle lamella, and the S3 that sits adjacent to the central cell lumen's tertiary layer. The tertiary layer, also called warty layer, consists of thin microfibrils and warts mostly composed of lignin. A model of the cell wall composition is given in Figure 1.10.

Wood is made up largely by cellulose, lignin and hemicellulose in various proportions (Eriksson *et al.* 1990). The three main carbohydrates are protopectin, hemicellulose and cellulose. Protopectin is a mixture of different acidic polysaccharides and is found along with hemicellulose, another polysaccharide, in the primary wall. Hemicelluloses are short, frequently branched homo- and heteropolymers and are made up of glucose and other sugars such as pentose or hexose and the precise composition depends on the tree species. The highest hemicellulose concentration in the cell wall is found within the S1 layer, making up approximately 20-30% of the softwood cells and contributes to the cell's elasticity.

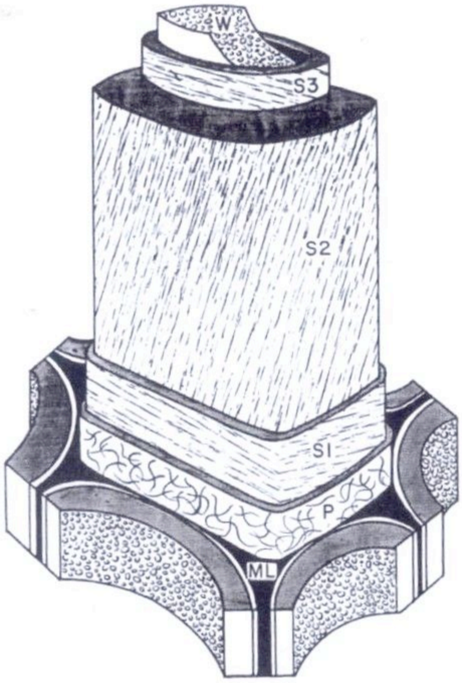


Figure 1.10 A model of several contiguous wood cells showing the organisation and microfibrillar orientation of the major cell-wall layers. The layers are identified from the middle lamella (ML) and outward as primary wall (P), the S1, S2 and S3 composing the secondary wall, and the warty (W) lining of the lumen surface. Courtesy of Professor J.J. Morrell (originally by Dr. W.A. Côté (1981) with the permission of the University of Washington Press).

Cellulose, like protopectin and hemicellulose, is a polysaccharide; although in structure it has only one linear homopolymer sugar unit, which is ß-D-glucose linked with ß-1-*O*-4 glycosidic bonds (Eaton & Hale 1993). In the S2 layer the highest concentration of cellulose is found, ranging between 40–50% for many wood species. The cellulose chains consist of many (approx. 100) molecules that are arranged in parallel to form microfibrils. These microfibrils are formed namely by crystalline cellulose linked with non-crystalline cellulose. If many microfibrils are connected with each other they form macrofibrils, leaving little spaces in between called interfibrilar spaces. Into these spaces not only water, but more importantly, larger molecules like hemicelluloses and lignin can be stored (Lüttge *et al.* 1989). Therefore, the encrusted lignin can act as a protective 'film' for cellulose and hemicellulose, making them not directly accessible to hydrolytic enzymes secreted by wood degrading fungi (Kirk & Farrell 1987; Wakeling 2003).

Lignin (lat. Lignum = wood) is an amorphous, thermoplastic and three-dimensionally connected aromatic polyphenolic polymer, formed by condensation of three types of propane units (Eaton & Hale 1993) and is the greatest integral part of the lignified cell wall (Payen, 1838 "l`incrustation ligneuse"). The highest lignin concentration is found in the middle lamella but it is also common in the S2 layer as it forms most of the cell wall.

The three phenylpropane units from which lignin is constructed are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The chemical composition of lignin varies between softwoods and hardwoods, both containing cross-linked 4-hydroxy-3-methoxycinnamyl (coniferyl) and some *p*-hydroxycinnamyl (coumaryl) alcohol (Eriksson *et al.* 1990; Reading *et al.* 2003), but hardwood lignin additionally contains 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol (Eriksson *et al.* 1990).

The enzymes required to initialise lignification are represented as water-soluble glycosides (e.g. Coniferin = sugar + precursor coniferylalcohol) within the cytoplasm (Holz-Lexikon 2003), where lignification normally begins at the end of primary cell wall growth. This process starts from the middle lamella towards the cell lumen but exactly how is still not fully understood (Higuchi 2006). The theory of statistical polymerisation of Karl Freudenberg (1886–1983) is mainly accepted whereby the biosynthetic, pre-built cellulose microfibrils, surrounded by the hemicellulose as an active surface, are necessary for lignification (Freudenberg & Neish 1968).

1.4.2 Durability classes

Wood, in general, is a durable, organic, natural and sustainable product and is a well-recognised material that can be used to enhance the standard of living. It can, for example, find its applications in building houses, producing furniture, creating arts, games, toys and gadgets, making paper or as a source for heating to name a few of its uses. When used correctly, wood products can last for centuries and withstand biotic, chemical and physical attacks, factors that otherwise cause degradation of the materials produced.

Every timber species has a natural durability, shielding it from degradation factors for a limited time. The factor that probably has the biggest influence on the durability of timber being used as a building material is moisture. Failing to keep wood dry and allowing moisture to the wood for extended times, as discussed in the Weathertightness Section 1.2.1 and 1.2.2, requires other protective measures to be considered to prolong its service. These measures could include design, and/or timber treatments, as will be discussed in Section 1.8.

Most framing timbers have a moderate natural durability of 5-15 years, as measured on the basis of in-ground contact life expectancy of the heartwood of each species. Table 1.1 shows common New Zealand hardwood and softwood timbers and their respective durability class.

Table 1.1 Natural durability classes of NZ timbers. Excerpt from Page *et al.* (1997).

Class	Description	In-ground life expectancy (years)	Example species softwood	Example species hardwood
1	Heartwood of timbers of the highest natural durability	>25	Silver pine Totara	Robinia
2	Heartwood of high natural durability	15-25	-	Hard beech Mountain beech Red beech Southern rata
3	Heartwood of only moderate durability	5-15	Kauri[1] Muricata pine[1] Radiata pine[1] Strobus pine[1] Douglas fir[1] Macrocarpa[2] Rimu[2]	Hinau[1] Mangeao[1] Blackwood[1] Black beech[2]
4	Heartwood of low durability	<5	Corsican pine Ponderosa pine	Tawa Silver birch

[1]Lower end of expectancy range; [2]Upper end of expectancy range.

Not until the tree is 10-14 years is heartwood formed in *P. radiata*, and until the age of 20 it only has a volume share of 10% of the total tree mass. After 40 years, heartwood in *P. radiata* accounts for approximately 20-30% (Harris & Cown 1991).

New Zealand framing timber is produced predominantly from *P. radiata* sapwood. As for all sapwoods, this part of the tree is regarded as non-durable. Nonetheless, when processed correctly *P. radiata* is suitable for practically any building components and accepts, where needed, all levels of chemical treatment.

1.4.3 Natural recycling of wood

Like every other organic material on earth, wood will be subject to degradation at some stage, no matter how well it is protected. Wood decay fungi are the natural ‘threat’ to the timber, be it fallen branches, tree stumps, whole trees (living or dead) or timber products in service. Being subject to degradation is part of the life cycle of the tree. The fungi use the timber as a nutrient source. Subsequently, through these nutrients, fungi are able to develop a fruiting body and reproductive spores to be distributed to colonise a new nutrient source. Additionally, and probably even more important for the tree's lifecycle, many homobasidiomycete fungi can 'give away' some of the consumed nutrients at the same time to another tree (Smith & Read 1997), either in making available the mineralised organic matter 'compost' to the trees or in the form of a symbiosis called mycorrhiza where the tree roots are connecting with the fungal hyphae. In this relationship between tree and fungi both can enhance their nutrient accessibility, necessary for growth development. Not easily available nutrients like nitrogen, phosphorus and potassium are passed on to the tree and carbon is transferred to the achlorophyllous fungi. Therefore, fungi help 'composting' dead organic matter, in the forest or in constructions, while for the former supporting trees to grow to their full potential, ensuring to serve again as wood products.

1.4.4 Decay establishment

Fungal spores or hyphal fragments are omnipresent in the air as wind is one of the major ways for their distribution to explore new grounds for nutrients like wet framing timber. Other means of spore distribution can be water, animals or insects and human (Rypáček 1966; Jennings & Lysek 1996; Goodell 2003). Once a suitable nutrient source is located and the right conditions prevail (Section 1.5), the spores germinate and produce very fine and thin hyphae that start to colonise the timber.

The primary and easiest path of colonisation of wood, no matter which type of fungal species, is usually by following ray cells or axial parenchyma cells. These elongated and thin parenchyma cells have fewer dividing cell walls to overcome in longitudinal direction and many carbohydrates are stored within them that provide the fungus with energy. Movement along the grain is always quicker than across, as these cells had the primary function of water and nutrient supply/transport in the living tree. Another example to illustrate this point can be that water or preservative uptake is much higher along the grain than through radial tracheids and, if no pressure is applied, liquids will travel further in the narrow latewood cells because of capillary forces.

Once a fungus has established itself within the lumenae of the parenchyma cells, hyphae then spread into tracheid lumens (or fibres and vessels) as can be seen in Figure 1.11, and further colonisation takes place via pits (Figure 1.12) or less often by thin bore hyphae producing tunnels (Figure 1.13) (Daniel 2003).

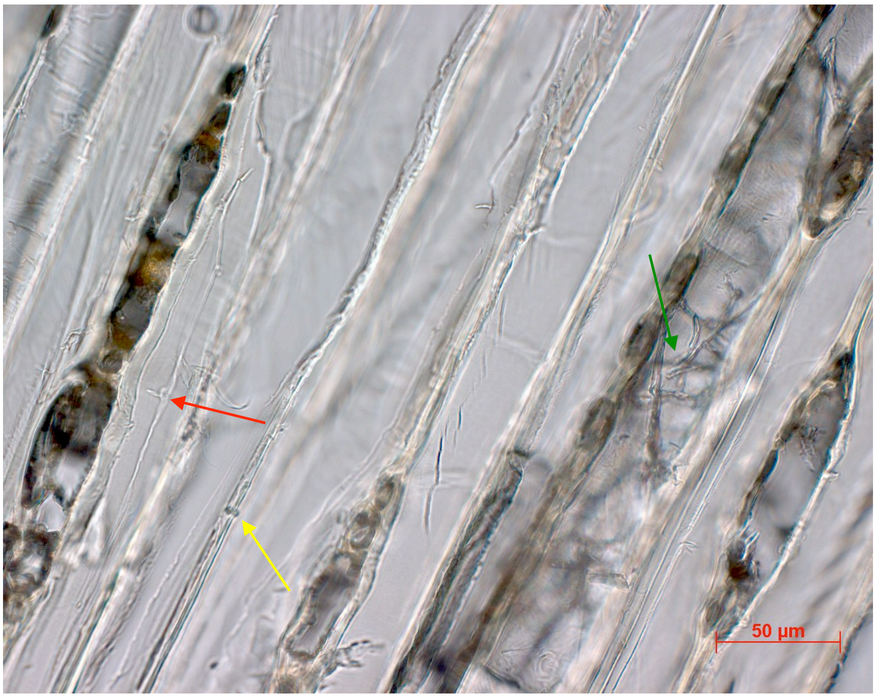


Figure 1.11 Picture showing hyphal growth (green arrow), clamp connection (red arrow) and tunnel (yellow arrow).

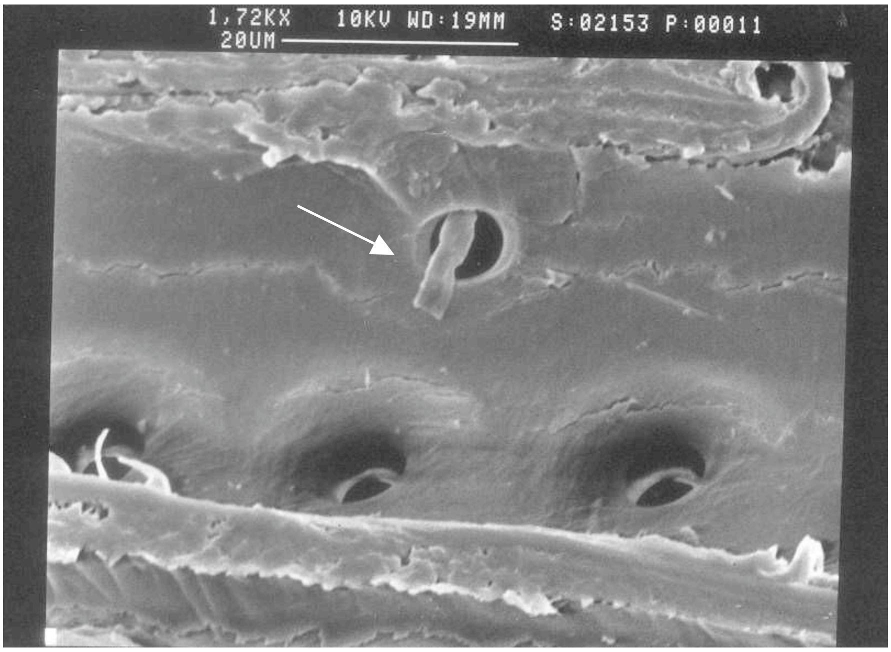
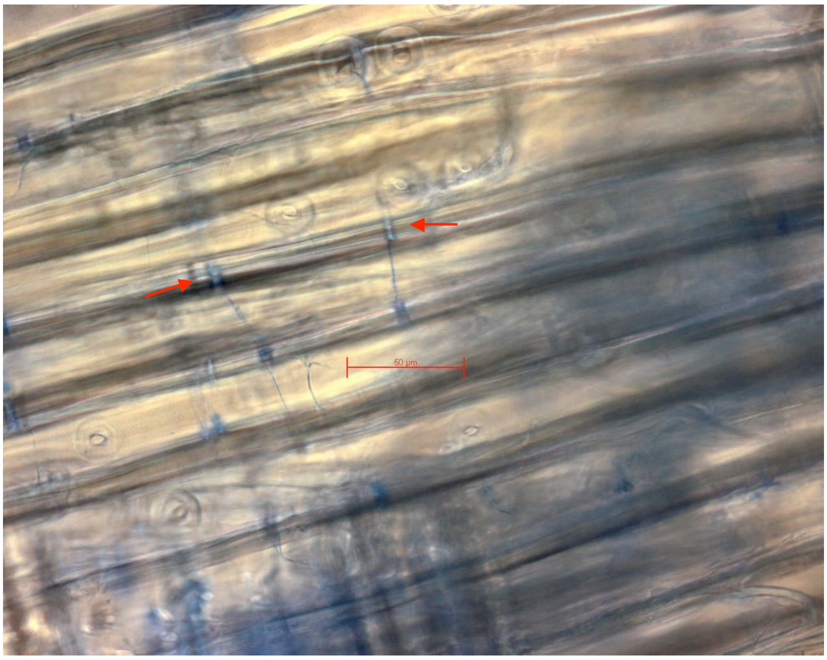


Figure 1.12 SEM picture of P. *radiata* tracheid showing hyphal movement through a pit (arrow).



Firgure 1.13 Picture showing lactophenol-blue stained bore hyphae producing tunnel (red arrows).

The mechanism for the bore hyphae to initialise the cell wall breakthrough is not fully understood, but the involvement of mechanical, chemical and enzymatic processes is assumed (Goodell 2003; Schmidt 2006).

For example, once, brown rot decay fungi have established themselves within the lumenae, the fungus releases "a glucan layer which coats the wood cell walls, and this 'hyphal sheet' helps to bind the hyphae to the S3 layer of the wood cell wall" (Goodell 2003, after Illman & Highley 1989), from where the actual degradation of the substrate wood begins.

1.4.5 Degradation of wood

The degradation of wood in service can simplistically be divided into four stages. According to Rayner *et al.* (1985), these stages are arrival (as described previously in Section 1.4.4) of the fungus, establishment on a suitable substrate (suitable moisture content i. e. > Fibre saturation point (30% moisture content (MC)), described in Section 1.5.1), exploitation of the substrate and exit of the fungus.

Huckfeldt & Schmidt (2006a) further divided the decay establishment stage into a) beginning of depolymeristion of holocellulose and at the same time b) making provisions for possible changing conditions.

Generally, the decay fungus uses all nutrients to build infrastructure in the colonised timber. The degradation is through the release of hydrolytic and oxidative enzymes (Section 1.5.7) in the presence of water, enabling the fungus to absorb the now diluted sugars for metabolism (growth and reproduction) (Jennings & Lysek 1996). At this stage of decay establishment, effort is being made towards production of arthrospores (resting spores) that are more resistant to heat, dryness and wood preservatives compared to fungal hyphae (Schmidt 2006). These reproductive spores are often produced and stored within the surface mycelium or within the first tracheid cells in the outer wood layers (Huckfeldt & Schmidt 2006a). Commonly, an attack from wood decay fungi stays unnoticed until this stage, as most activity is within the timber and is difficult to determine with the naked eye, but sometimes fungal strands (cords) or mycelium grow on the surface that indicate fungal presence (Figures 1.14 & 1.15).

Figure 1.14 *P. radiata* wall stud showing early signs of decay establishment on the surface, indicated through formation of white mycelium (arrows). Source: Prendos Ltd., Auckland, NZ (2007).

Under extended favourable conditions for the fungus, e.g. if constant high water content within the wall framing is not detected, it will carry on with the depolymerisation of the cell wall constituents holocellulose and lignin until the substrate is fully exploited. At this stage of advanced degradation, most decay fungi produce plenty of surface mycelium and strands exploring the surroundings for other suitable nutrient sources, e.g. progress of decay into the second story of a house, and are able to grow over building materials other than timber, e.g. building foil or fibre cement cladding (Figure 1.15). For one decay fungus in particular, *Serpula lacrymans*, it is known that it can even penetrate through masonry (Schmidt 2006). Nonetheless, nearly all decay fungi will start to produce either fruiting bodies or arthrospores to ensure reproduction (Section 1.3.1) and distribution of the fungal species. As wood is low in protein, the fungus will use its own protein sources by reabsorbing the formerly produced hyphae to provide the nutrients needed to establish fruiting bodies and subsequently spores for distribution. However, the exact point in time when and under which circumstances fruiting bodies are developed is still uncertain (Huckfeldt & Schmidt 2006a).

During the exit stage millions of (basidiomycete-) spores are released from the fruiting bodies (Figure 1.7 Section 1.3.1.3) into the surrounding air, leaving behind a highly degraded timber (Figure 1.16).

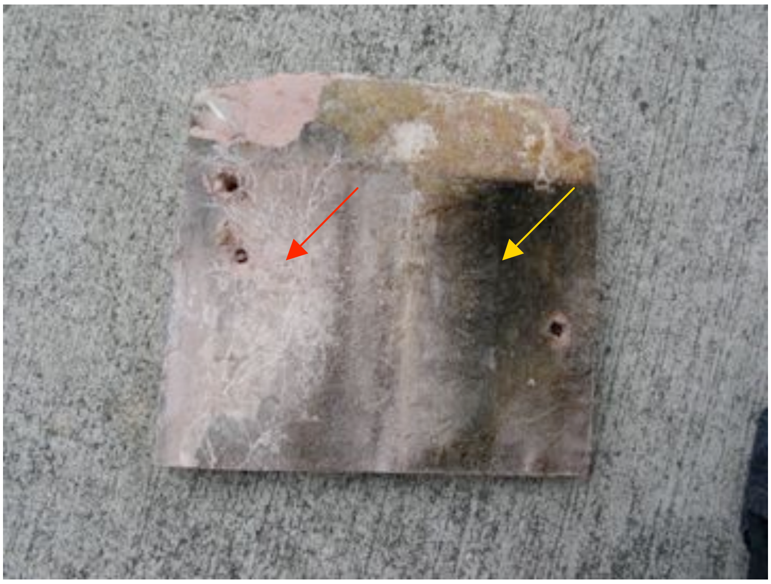


Figure 1.15 Two types of mycelium advancing on fibre cement cladding material. White strands on the left hand side (red arrow) and brownish-black mycelium on right hand side (yellow arrow).

Figure 1.16 Corner wall stud heavily degraded and exploited for nutrients by decay fungi.

1.5 Environmental implications on fungal growth

Considering the colonisation of construction timber through wood decay fungi, it is apparent that there are multiple factors that have an impact on fungal establishment, development and survival. This section outlines the most important physiological parameters necessary for optimal fungal growth. Most important is water, which in the right range is essential for fungal growth and survival, and especially in terms of the fungal metabolism, but not too much that it restricts access of fungi to oxygen. In most aspects discussed in this section, water is a key parameter interacting with the other physiological factors, i.e. water‑temperature relationships, nutrient transport or the release of enzymes into the substrate.

1.5.1 Moisture content of wood

The moisture content (MC) of wood is given as a percentage based on the oven dry weight of the wood species. For timber in all its uses, the critical moisture content is around the fibre saturation point (FSP), which is at approximately 30% (Eaton & Hale 1993). Generally, below FSP, all water is tightly bound to the polymers of the cell walls and is unavailable to most fungi (Rypáček 1966; Zabel & Morrell 1992, Morrell 2002). Only when the water content in wood rises above the FSP in the form of free water in the wood cell lumina and/or other cavities does timber in use get susceptible to decay. Therefore, it is commonly accepted that wood kept dry will in most cases be protected from fungal attack. However, this might not always be the case especially if the timber was pre-infected by fungal hyphae or resting spores that can withstand longer periods of drought (dormant vegetative state) (Carll & Highley 1999; Nicholas & Crawford 2003) and might start to colonise timber again at MC below FSP. Further, Huckfeldt & Schmidt (2006a) recently pointed out that many decay fungi found in buildings could colonise timber well below FSP, in the MC range of 18 to 28%. One example could be the true dry rot fungus *Serpula lacrymans* that thrives best on MC between 25-55% (Viitanen & Ritschkoff 1991; Bech-Andersen 1995).

To give an indication of marginal and critical moisture content values for timber in construction, the following can be a guideline (based on Findlay 1953, Rypáček

1966, Scheffer 1973, Viitanen & Ritschkoff 1991, Zabel & Morrell 1992, Nicholas & Crawford 2003, Mai *et al.* 2004, Huckfeldt & Schmidt 2006a):

- 16% MC minimum requirement for fungal (e.g. mould) growth;
- 20% MC widely accepted minimum threshold below which decay is prevented;
- 20-30% MC range over which it is difficult to be certain if decay gets established or not;
- > 30% MC (FSP): decay inevitable;
- 40-80% common MC range for aggressive decay.

The optimum moisture ranges for growth for some building decay fungi, according to the literature, are given in Table 1.2, Section 1.5.3, together with their optima temperature ranges, as the interaction of these two factors are important for fungal decay in the field (Brischke & Rapp 2007), and in terms of the habitat "building" (Schmidt 2007).

A moisture content in wood above the optimum for a specific fungus usually slows down the decay rate or halts it completely (Zabel & Morrell 1992; Råberg *et al.* 2005; Schmidt 2007) due to reduced oxygen levels available within the cell walls, as will be discussed later in Section 1.5.5. To give an example in this context, the building decay fungi *Coniophora puteana* and *Gloeophyllum sepiarium* need minimum free oxygen within the cell lumina of 15 and 20%, respectively (Rypáček 1966). Additionally, Findlay (1953) and Nicholas & Crawford (2003) described that this free oxygen can differ between wood species, as it will be higher in very light wood and lower in very dense wood.

In terms of construction timber or generally timber in use for interiors or exteriors, the best approach is to keep the wood dry, or if moisture builds up periodically at stages that it can dry out again. Factors that can cause increased moisture levels for timber in buildings are often related to one or more of the following conditions according to Ridout (2000), Huckfeldt & Schmidt (2006a) and as further discussed under common leakage problems (Section 1.2.3):

- **Condensation**, i.e. on window glass and frame, water vapour emissions through living activities such as cooking, taking a shower or heating, non-insulated cold water pipes.
- **Faults at water conducting pipes**, i.e. inappropriate installation, blocked water drains, defects at water mixers.
- **Ground water**, i.e. no drainage, defective water barrier in the foundation, root systems of trees that penetrate the building envelope, floods.
- **Rainwater**, i.e. no maintenance of the building envelope and storm water pipes such as defects of roofs, gutters, cracked paint on weatherboards or defects of cladding materials, windows and doors.

In summary, the most important questions omnipresent when viewing construction timber are whether the critical moisture limits were conductive or hindering to decay at a certain point in time, how moisture fluctuations and duration influenced the decay rate, and how deep the water was able to penetrate into the timber?

1.5.2 Humidity

Wood is a hygroscopic material and is therefore responsive when exposed to changes in relative air humidity (RH)(Eaton & Hale 1993). As there is no constant atmosphere with ever changing temperature and RH, wood is constantly (as every other hygroscopic material) trying to achieve a stable equilibrium moisture content (EMC) condition resulting in either gaining moisture (adsorption) or losing moisture (desorption). The latter however takes longer to achieve, resulting in higher EMC values than for adsorption at the same RH and temperature. In terms of wood technology, especially in the field of wood drying, this phenomenon is called hysteresis, defined as the lag in moisture content/sorption curves (Pasanen *et al.* 2000, Holz-Lexikon 2003).

The EMC of timber in constructions (wall framing) usually ranges from 6 to 14% but it can reach EMC's of 17-20% under high humidity conditions (Morrell 2002). 20% EMC in *P. radiata* D. Don would compare to approximately 90% RH at 15°C according to Kininmonth (1991) and at 70% RH it would be around 14%

MC. 100% RH at 15°C would reflect a MC of very close to FSP (28%) for *P. radiata* (Kininmonth 1991). Orman (1955, in Kininmonth 1991) calculated that the general EMC for *P. radiata* sapwood stored outside and under cover in the North Shore Auckland was 14.3% in summer and 20.8% during winter with a yearly mean of 16.7%. In perspective, according to the 2003 version of the New Zealand Standard for Timber and Wood-based Products for Use in Buildings (NZS 3602:2003), the MC range for enclosed framing is given at 12 to 24% at the time of enclosure.

Other parameters that can further influence the EMC are wood species (i.e. their respective density) and wood preservatives, which can increase the EMC by approximately 1% (Kininmonth 1991).

1.5.3 Temperature

The temperatures at which fungi are capable of actively growing and reproducing within or on a given substrate vary and are termed physiological temperatures. Generally, each fungus has three cardinal growth temperatures: minimal (growth begins), optimal (best growth) and maximal (growth ceases). Of highest interest in regards to wood decay fungi is the optimal level, as it is the temperature where the fungus is most competitive and causes the highest damage.

Some authors refer to wood decay fungi as *mesophilic* fungi, meaning they grow best in temperature ranges between 15 and 40°C (Jennings & Lysek 1996; Zabel & Morrell 1992). On the other ends of the spectra of this temperature range, fungi are either referred to as being *psychrophilic* (cold-loving; growth at 0 to 17 but incapable of growth at 20°C) or *thermophilic* (heat-loving; growth above 50°C).

For most wood decay fungi, growth has been recorded between 12 and 45°C (Zabel & Morrell 1992; Rayner & Boddy 1988) and their optimum temperature range is given between 20 and 35°C (Grosser 1985; Rayner & Boddy 1988; Huckfeldt & Schmidt 2006a). Beyond this temperature range, wood decay fungi go into a dormant state and survive colder or warmer temperatures by forming resting or dehydrated structures. Some fungal species can live, survive and degrade wood at the lower temperature range < 0-10°C (fungi isolated in the Antarctica) (Duncan 2007) and others are still active at higher ranges up to 65°C (fungi found in decomposing compost or wood chip piles) (Jennings & Lysek 1996; Zabel & Morrell 1992).

Usually, exposure to temperatures above the growth maximum or below the growth minimum are lethal to most wood decay fungi but killing is further dependent on the length of exposure to those temperatures and the humidity present. Many have reported that it takes much longer to kill a fungus under dry conditions or in dry wood than it does take under moist conditions (Mizumoto 1951; Snell 1923; Rayner & Boddy 1988; Jennings & Lysek 1996; Huckfeldt *et al.* 2005). For example, the brown rot decay fungi *Gloeophyllum trabeum* and *G. sepiarium,* often found in exterior wooden structures (Nicholas & Crawford 2003), were cited in a review by Zabel & Morrell (1992) to resist a heat treatment of 60°C for 12 hours but had been killed at 66°C for 75 minutes using colonised green timber (MC >FSP). A recent study by Huckfeldt & Schmidt (2006a) where the same fungi were used, this time with dry timber (12% MC), demonstrated the lethal temperature was far higher and slightly above 95°C for 4 hours heat treatment.

The lethal temperature of a specific fungus is very important especially if heat treatments, which are sometimes used as remediation practices in effected buildings, are applied. In Denmark, for example, heat treatments are sometimes successfully used against the true dry rot fungus *Serpula lacrymans* (Rudolphi 1995; Steinfurth 1999) but generally it is regarded as unsuitable for most buildings because it is often impossible to achieve the very high temperatures (<90°C for 4 hours) required to kill the fungus without damaging the building (Huckfeldt & Schmidt 2006a).

Growth rates, optimal temperature ranges, lethal temperature and optimum moisture content ranges for the most important decay fungi identified in this PhD thesis research are given in Table 1.2 according to test results of various authors.

Table 1.2 Decay fungal growth parameters.

Fungus	**Temperature (C°) range optimum on artificial media**[2,3,4,5,7]	**Lethal temperature in dry wood**[2,3,5] **in hours**		**Moisture range**[2,4,5,7] **(%)**		**Growth rates**[6] **(mm day^{-1}) on artificial media at near optimum temperature**	**Decay rates (%) after 10-12 weeks in untreated pinus**	
		(C°)	**(h)**	**Min.**	**Opt.**		**radiata**[1]	**& sylvestris**[2,6]
Oligoporus placenta	25-28	>80	(4)	-	-	6.3	47.2	62.0
Antrodia sinuosa	25-31	>65	(3)*	>25	>35	-	58.8	20.8
Gloeophyllum sepiarium	28-38	>95	(4)	>26	>46	-	20.3	59.0
Gloeophyllum trabeum	30-38	>95	(4)	>25	>45	6-11.4	14.6	59.0
Coniphora puteana	20-32	75	(4)	>18	>36	6.7	34.9	27.5
Serpula lacrymans	15-23	70	(4)	>17	>45	5.5	11.2	49.0

The data for this table was collected and summarised after the following authors:
[1] PhD research data Dirk Stahlhut;
[2] Huckfeld & Schmidt (2006a); Schmidt & Huckfeld (2005) and Huckfeldt *et al.* (2005);
[3] Schmidt & Moreth (2003);
[4] Zabel & Morrell (1992);
[5] Cartwright & Findlay (1958) and Grosser (1985);
[6] Eslyn (1986) and Wälchli (1977);
[7] Viitanen & Ritschkoff (1991);
* Lethal temperature for surface mycelium.

Temperature not only influences the growth of fungi but also fruiting body formation, spore germination and/or enzyme activity (Grosser 1985). In ***Fungal Morphogenesis***, Moore (1998) outlined that basidiomycete fungi may require a downshift in temperature to fully develop a fruiting body but that the functional significance remains unknown. Jennings & Lysek (1996) reported a laboratory study where a temperature drop of only 1°C was sufficient to trigger, for example, sporulation of some fungi but the fungi were not named in the paper. As fungi in nature are exposed to temperature oscillations, it seems plausible that the key developmental processes depend on temperature-sensitive proteins (Moore 1998).

1.5.4 Light and UV

Generally, light was considered not to be important to wood decay fungi and can even harm the growth of active hyphae if exposed to wavelengths of light 390 nm or more as represented by ultraviolet (UV) radiation (Zabel & Morrell 1992). However, it has been shown that fungi have developed sophisticated relationships with the daily light-dark cycles, particularly with respect to the initiation of differentiation (Jennings & Lysek 1996; Yang & Illman 1999; Moore 1998). Plunket (1956), for example, showed that light and aeration promoted cap development from the stem of *Polyporus brumalis* and Moore (1998) reported that short light exposure initiated pore formation of *Phellinus contiguus* in culture.

Visible light is represented through different colours (in nature sometimes made visible to the naked eye as in a rainbow) and their corresponding wavelength. Violet light, for example, ranges from 390-430 nm (shorter wavelengths) and red light from 620-780 nm (longer wavelengths). The part of the light that has the best effect on fungal differentiation is blue light with shorter wavelengths of 460-490 nm (Jennings & Lysek 1996), whereas longer wavelengths, such as red light, have no effect on fungi and are often used in experiments in which it is necessary to inoculate and grow fungi in culture in an environment which for them is ‘dark’. However, a recent review outlined that the fungal kingdom is not ‘blind’ to light above 600 nm and that, for example conidiation of *Aspergillus nidulans,* is sensitive to both red and blue light (Herrera-Estrella & Horwitz 2007; Purschwitz *et al.* 2008).

Fungi respond and interact with the environmental signal of light in various ways, like simply reorienting the direction of growth (which way is up or down), impending stress or, as mentioned above, triggering the onset of reproduction. As the bulk of the fungal mycelium grows mainly within the substrate (soil, wood, living tree), the fungus reacts to light that it has reached the (irradiated) surface and is stimulated to either differentiate or ‘turn around’. One example with respect to building decay fungi are the brown rot species of *Gloeophyllum* spp. which predominately decay the inner wood cells first and only reach the surface if all nutrients are depleted. Another response to light by fungi might explain the pigmentation of spores and many fruiting bodies to minimize the killing effect of heavy irradiation. In the fungus *Neurospora crassa* (a fungus predominately under investigation in light-response studies), blue light triggers the synthesis of photo-protective pigments (the carotenoids), spore formation, entrainment of the circadian clock (physiological rhythms) and growth towards a light source (phototropism) (Linden *et al.* 1997).

Fungi appear to sense blue light but the required quantities are very low, i.e. Jennings & Lysek (1996) in their book ***Fungal Biology: Understanding the fungal lifestyle*** described the low light requirements below those "of the full moon on a clear night" (Jennings & Lysek 1996), which is represented after Schmidt (2006) with 230 µW m^{-2} (200 µW m^{-2} = about 25% of midday light intensity at midsummer in Rotorua, NZ (Bulman *et al.* 2004)). This sensing of light is via a receptor which is an unique transcription factor in filamentous fungi with a flavin chromophore (riboflavin) that activates its targets in a light-depending manner, today known as the white collar (WC) complex (Herrera-Estrella & Horwitz 2007). The WC complex is a protein complex consisting of two proteins, WC-1 and WC-2. WC-1 is a protein with a flavin-binding domain and a zinc-finger domain protein and interacts with WC-2, another zinc-finger domain protein. Together they operate as a photoreceptor and transcription factor for blue light responses i.e. in *N. crassa* (Herrera-Estrella & Horwitz 2007; Corrochano 2007). Corrochano (2007) further outlines that proteins similar to WC-1 and WC-2 have been described in other fungi, suggesting a general role for the WC complex as a fungal receptor for blue light.

1.5.5 Oxygen

Most fungi are aerobes and oxygen is a prerequisite for their hyphal extension and growth with the insertion of precursors (i.e. long-chain polymers, predominately chitin for eumycota) and maintenance of polarity at the hyphal tip dependent on metabolic energy, which is provided by the respiratory oxidation of carbon compounds. Another by-product in this reaction is water. Zabel & Morrell (1992) outlined that the amount of oxygen consumed by aerobic fungi is directly related to the amount of carbon dioxide produced; its ratio expressed as the respiration quotient, which has been used to monitor decay development, compare growth rates of fungi on various substrates and to evaluate wood preservatives.

Oxygen in the atmosphere is provided at about 21%, but fungi need far less to flourish as discussed in this section. When considering the degradation of wood or in other words the development of fungi on a substrate (hyphal extension), moisture content and oxygen exist in a close relationship. As discussed earlier, the optimum MC range is 40-80% for most basidiomycete decay fungi studied (Zabel & Morrell 1992) and it is generally considered that 20% residual air volume (of void volume) in wood is close to the minimum level needed for wood decay basidiomycete fungi (Nicholas & Crawford 2003; Zabel & Morrell 1992; Rypáček 1966). As an exception, soft-rot fungi have a greater tolerance for low oxygen levels in wood, which explains why they are capable of decaying wood products with high MC levels or that are fully saturated with water (Nicholas & Crawford 2003). However, wood with very high moisture content in buildings might be degraded by all types of decay fungi as the framing has a small cross-section and is surrounded by plenty of air within the wall cavity.

A higher demand for oxygen by fungi is needed when hyphal growth ceases and reproduction is initiated, also known as differentiation. Here, the production of fruiting bodies and spores require far higher protein and nucleic acid synthesis which requires a higher energy demand by fungal metabolism to produce e.g. wall material (Jennings & Lysek 1996). This explains why most fungi form their fruiting bodies at or near the surface of their substrate where the necessary oxygen demand can be matched. Therefore, oxygen influences all stages of fungal development and differentiation.

1.5.6. Nutrients

Wood decay fungi not only need organic compounds and water for growth and development but inorganic nutrients as well. Moisture and/or water contains dissolved inorganic nutrients; when the ground, stucco or stone materials are in contact with the framing timber, they provide the mineral nutrients and phosphates necessary for fungal growth and development (Huckfeldt & Schmidt 2006a). Further, fungi can play an active part by directly extracting and metabolising required nutrients from the building environment like plaster, fibreglass and the ground (Bech-Andersen 1987) and especially wood. All nutrients essential for fungal growth can be divided into macro- and micro-elements:

Carbon (C), nitrogen (N), phosphorus (P), sulphur (S), magnesium (Mg) and potassium (K) are the major elements required by fungi for growth (Jennings & Lysek 1996; Zabel & Morrell 1992; Hawker 1950) with the first four making up most of the structural components of the fungal cell or hyphae. Magnesium is required as the activator of a number of key enzymes, while potassium is needed to guarantee the appropriate ionic environment for enzyme functioning (see Section 1.5.7 for key enzymes). Together these elements contribute to the regulation of the internal osmotic hyphal potential (Jennings & Lysek 1996). Additionally, in small quantities, magnesium and potassium have a great effect on sporulation (Hawker 1950).

The other elements required for fungal growth are calcium (Ca), iron (Fe), copper (Cu), manganese (Mn), zinc (Zn) and molybdenum (Mo), but there is still uncertainty if this is a complete list. All except calcium are activators of enzymes (Zabel & Morrell 1992; Jennigs & Lysek 1996), while calcium is thought to be involved in cell signalling (Jennings & Lysek 1996). In the literature, these elements are also known as the trace elements, as only minute amounts are required by the fungi to grow (Hawker 1950).

The most qualitatively and quantitatively nutritional element all fungi depend on is organic carbon, which is produced through photosynthesis. Organic carbon is a major part of living plants, animals and microbes. In plants, it is stored in cellulose and lignin and for animal and microbes, in chitin and keratin. Fungi are able to degrade all these molecules with significant success (Jennings & Lysek 1996).

Another element, nitrogen, has been found within the mineral nutrients to be needed in the largest quantity by wood decay species (Rayner & Boddy 1988) and levels within wood have been reported to range between 0.03-0.13% of the dry weight of wood (Hudson 1986; Ridout 2000). Ammonium is the preferred inorganic source of nitrogen for wood decay fungi as most of them are unable to utilise nitrate (Rayner & Boddy 1988; Jennings & Lysek 1996; Zabel & Morrell 1992). All the necessary nitrogen needed for growth development by wood decay fungi is entirely found in the wood/timber products through the present wood proteins (and subsequently the amino acid composition) and other nitrogenous compounds (Kininmonth & Whitehouse 1991; Zabel & Morrell 1992). As we know that nitrogen is beneficial to fungal growth, Weber (1996) in Huckfeldt & Schmidt (2006a) further report that higher nitrogen-, phosphate- and potassium levels can contribute to wood degradation being more severe than with lower levels.

The forms of the element phosphorus that are easily digested by fungi are orthophosphate or organic phosphorus compounds found within the remains of the dead wood and, according to Jennings & Lysek (1996), the living tree is only able to absorb and store orthophosphate. Further, they state for sulphur: "The source of sulphur for fungi is either inorganic, the preferred form being sulphate, or low molecular weight organic molecules, such as the amino acid methionine." For *P. radiata*, to give an example, concerning quantities of amino acids contained in softwood, Scurfield & Nicholls (1970) found 16 different amino acids with methionine being detected at a level of 1.4% of the total.

In *P. radiata*, according to a study by Orman & Will (1960), the distribution levels within the stem-wood of nitrogen, phosphorus and potassium increased from heartwood towards the outer sapwood. Zabel & Morrell (1992) supported these findings and suggested that due to changing nitrogen occurrence levels within the same timber, this might account for variability in results of the standardised decay test. However, as nitrogen can be reabsorbed from parts of the fungus that are no longer required when decay progresses (Cowling & Merrill 1966), the variation of nitrogen levels might be overcome by the fungus. Literature from more than 30 years ago stated that the younger the tree, the higher the content of N, P and K (Orman & Will 1960; Madgwick *et al.* 1977) but no newer studies could be found on this point.

1.5.7. Enzymes

Most importantly for the degradation of wood by decay fungi are the enzymes that are secreted/released from the hyphal tip(s) into the substrate. These enzymes are called exo-enzymes or extracellular enzymes and have the task to bring the solid substrate i.e. wood into a dissolved form that can be absorbed by the fungal hyphae to cater for the various nutritional and energetic requirements for growth. Further, there are intracellular endoenzymes present within the living cells to guide fungal metabolism (Rypáček 1966). The main enzymes that are produced by brown-, white- and soft-rot fungi and involved in cellulose, hemicellulose and lignin degradation are discussed in this section.

Cellulose as the major nutritional carbon source in the wood cells is hydrolysed by cellulolytic microorganisms e.g. fungi producing extracelluar enzymes generally (but inaccurately) referred to as *cellulases,* which are hydrolytic enzymes catalysing the reactions on the cellulose polymer, which hydrolyse i.e. break apart the long cellulose chains into shorter cellodextrines (glucose polymers) like cellotetraose, cellotriose and cellobiose and finally into glucose chains that can be used by the fungus (Rypáček 1966). Both hydrolytic enzymes as well oxidative enzymes take part in cellulose degradation (Eriksson & Wood 1985).

The set of hydrolytic (cellulolytic) enzymes that successfully degrade crystalline cellulose are composed of the following enzymes according to latest review by Baldrian & Valášková (2008):

- endo-1,4-β-glucanases (EC 3.2.1.4) which randomly attack and split $\beta(1\rightarrow4)$-glucosidic linkages over the cellulose chain.
- exo-1,4-β-glucanases (EC 3.2.1.91) that splits off either cellobiose or glucose from the non-reducing end of the cellulose.
- 1,4-β-Glucosidase (EC 3.2.1.21) hydrolysing cellobiose and other water-soluble cellodextrines to glucose.

To fully degrade the cellulose polymers, additionally oxidative enzymes are required. The enzymes involved were described by Eriksson *et al.* (1990) after extensive studies of the white rot fungus *Phanerochaete chrysosporium*, one of the most studied lignin degrading fungi (Kirk & Farrell 1987, Kersten 1990):

- Cellobiose: quinone oxidoreductase (cellobiose dehydrogenase) reduces quinones and phenoxy radicals in the presence of cellobiose, which is oxidised to cellobiono-δ-lactone (Westermark & Eriksson 1974a,b: In Eriksson *et al.* 1990; Ander *et al.* 1990; Baldrian & Valášková 2008).
- Cellobiose oxidase oxidises cellobiose and higher cellodextrines to their corresponding-onic acids using molecular oxygen (Ayers *et al.* 1978: In Eriksson *et al.* 1990; Baldrian & Valášková 2008).

Depending upon the wood species, about one-quarter of the wood cell wall material consists of hemicelluloses (heteropolymers) (see Section 1.4.1) consisting of different pentose and hexose residues, often having branched chains and are typically composed of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid (Eriksson *et al.* 1990). The specific composition of hemicelluloses is different between hardwoods and softwoods and within wood species, as discussed in Section 1.4.1. To hydrolyse the hemicellulose heteropolymers, different extracellular enzymes are necessary and are, for example, either galactoglucomannanases to cleave the hexosane mannan (glucomannan) in softwoods, or xylanases to cleave the pentosane xylan in hardwoods (Rypáček 1966). The main hydrolytic enzymes involved are presented in Table 1.3:

Table 1.3 Hydrolytic enzymes involved in hemicellulose degradation.

Xylanolytic enzymes		**Mannanolytic enzymes**	
endo-1,4-β-xylanase	(EC 3.2.1.8)	endo-1,4-β-mannanase	(EC 3.2.1.78)
β-xylosidase	(EC 3.2.1.37)	β-mannosidase	(EC 3.2.1.25)
α-glucuronidase	(EC 3.2.1)	β-glucosidase	(EC 3.2.1.21)
α-L-arabinofuranosidase	(EC 3.2.1.55)	α-galactosidase	(EC 3.2.1.22)
acetylesterase	(EC 3.1.1.6)		

Lignin is the third wood cell wall component that is actively degraded by decay fungi to cause a white rot through the secretion of extracellular oxidases and peroxidases, which most brown rot fungi do not produce. The study of these fungi with regard to their biochemistry has been conducted for decades; Bavendamm (1928) was the first to divide brown- and white- rot fungi by their ability to produce oxidizing enzymes or not. Campbell (1930, 1931, 1932) took this one step further by placing the white rot fungi into three different groups:

I) Fungi that degrade lignin first, then cellulose (greater oxidases than hydrolases) (e.g. *Phellinus pini*).
II) Fungi that degrade cellulose first, then lignin (greater hydrolases than oxydases) (e.g. *Armillaria mellea*).
III) Fungi that degrade cellulose and lignin simultaneously (e.g. *Inonotus hispidus*).

This grouping still exists today but is referred to as selective (also called sequential decay) and simultaneous degradation (Otjen & Blanchette 1986) (see Section 1.6.1).

Lignin in wood forms an amorphic complex with hemicellulose surrounding the cellulose, a barrier that must be broken in order to give enzymes access to the cellulose (Kirk & Farrell 1987; Eriksson *et al.* 1990). The chemical composition of lignin varies between softwoods and hardwoods, both containing cross-linked coniferyl (4-hydroxy-3-methoxycinnamyl) and some *p*-coumaryl (*p*-hydroxycinnamyl) alcohol (Eriksson *et al.* 1990; Reading *et al.* 2003), but hardwood lignin additionally contains sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohol (Eriksson *et al.* 1990). To degrade lignin successfully, extracellular enzymes are released by the wood decay fungi, which produce free radicals by acting upon the lignin substructure aromatic molecules (Kirk & Farrell 1987; Levin *et al.* 2002; Reading *et al.* 2003). The major enzymes involved in lignin degradation are as follows:

➢ lignin peroxidase (LiP)	(EC 1.11.1.14)
➢ manganese-dependent peroxidase (MnP)	(EC 1.11.1.13)
➢ cellobiose dehydrogenase (CDH)	(EC 1.1.99.18)
➢ laccases	(EC 1.10.3.2)
➢ versatile peroxidase	

Additional factors attributed to free-radical generation by these enzymes are hydrogen peroxide (H_2O_2), oxalate, small molecule mediators, methyl transferases, and the plasma membrane redox potential (Kirk & Farrell 1987; Reading *et al.* 2003). These small chemical oxidisers or low molecular weight agents are thought to be involved in the initial steps of wood decay since it is generally believed enzymes, due to their large molecular size, are unable to penetrate the compact structure of sound wood tissues (Martinez *et al.* 2005). To mention one example discussed in the literature, and related to the initial depolymerisation of lignin, hypothesised to be involved is the ability to generate the hydroxyl radical ($OH^\bullet$), a strong biological oxidant, by CDH, which causes oxidation in the presence of H_2O_2 and catalytic metals such as iron or copper, also known as the Fenton reaction (Eriksson *et al.* 1990; Green & Highley 1997; Halliwell 2003; Reading *et al.* 2003; Martinez *et al.* 2005):

$$\underset{(Cu^{+})}{Fe^{2+}} + H_2O_2 \rightarrow \underset{(Cu^{2+})}{Fe^{3+}} + OH^{-} + OH^{\bullet}$$

It has been shown that through the synergistic release of enzymatic and non-enzymatic (re)action(s) of the decay fungi, these are the most efficient and significant wood degrading and recycling organisms of both softwoods and hardwoods. However, not all depolymerising systems can be attributed to fungi (brown-, white-, soft-rot) or generalised, as it has been reported/observed that there are often differences in mechanisms even among various strains of the same fungi (Green & Highley 1997, Irbe *et al.* 2001).

1.6 Micromorphology of wood decay

The most important factors influencing the different kinds of wood deterioration through white-, brown-, soft- rot and bacteria are presented in this section concerning micromorphology of wood decay. These four decay types are very different in their nature, following different pathways of depolymerising the holocellulose and lignin of timber in use. Special consideration is given to the decay mechanisms of brown-rot decay fungi, because they were found in this PhD research to play a major role in the degradation of framing and other wooden components of buildings that were investigated. However, there is usually not only a single type of organism present in decayed wood, it is more common to find multiple organisms present, presumably interacting or as successors of each other as illustrated in Figure 1.17.

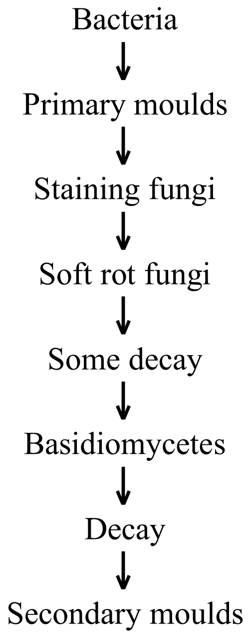


Figure 1.17 Model for the succession of micro-organisms in wood in ground contact based on colonisation of untreated Scots pine stakes; Source: Clubbe (1980; in Eaton & Hale 1993).

1.6.1 White rot fungi

White rot fungi can depolymerise all components of wood cell walls but are especially active in lignin degradation. They preferentially decay hardwoods and to a lesser extent softwoods and are known for their high activity in forest ecosystems in nature (Kim & Singh 2000), as well as causing extensive damage to wood constructions and building materials (Messner *et al.* 2003; Schmidt 2007).

The proportion in which the wood components are decomposed is not uniform and a great variation can be found between the different white rot fungi. For example, some species are specialised to primarily degrade lignin and hemicellulose, leaving behind nearly untouched cellulose (Blanchette *et al.* 1990; Eriksson *et al.* 1990)

All white rot fungi belong to the phylum *Eumycota*, and therein mainly to the class of *Basidiomycotina*, with several thousand species causing decay around the world (Gilbertson 1980). A few white rot fungi find their origin in the class of *Ascomycotina*. Both classes are often referred to as 'higher fungi'.

Hartig (1878) was the first to characterise white rot fungi by macroscopic and microscopic differences present in decayed wood. The bleached white colour of advanced decay and the ability to degrade lignin suggested that different enzyme systems were functioning in white rot and brown rot fungi. Following up on these early observations to differentiate between the two fungi, an identification method for wood rotting fungi in culture was developed (Bavendamm 1928; Davidson *et al.* 1938; Nobles 1958). This method relies on an extracellular oxidase reaction, where a coloured zone on agar indicates polyphenolic compound formation. The distinction between the two fungi subsequently is that the white rot fungi test positive for the oxidase reaction by forming a coloured zone while the brown rot fungi do not.

Latest research by Schmutzer *et al.* (2008) shows that the delignifying potential of white rot fungi can be directly related to their combined total peroxidase activity, and demonstrated that this is further related to overall amounts of lignin metabolised or solubilised. Based on their results, Schmutzer *et al.* (2008) suggested that white rot fungi could be classified depending on their ability to release peroxidase in greater or lower amounts to depolymerise lignin, into ‘fast’ white rot or ‘slow’ white rot fungi.

When examining timber samples degraded by white rot fungi, the wood can appear brittle, soft and spongy or separated into string-like fragments. Generally, the wood degraded by white rot fungi takes on a lightened and bleached appearance (Kim & Singh 2000) but also darkened and brown tinges and streaks can occur in early stages of decay (Eaton & Hale 1993). To achieve this, a moisture content far above the fibre saturation point has to be contained within the timber over extended periods (Goodell *et al.* 2003).

From microscopic and ultrastructural observation two main types of white rot fungi have been distinguished. Today, these types are classified (Otjen & Blanchette 1986) by the order they degrade the different amounts of wood components and are directly related to the enzyme activity classification by Campbell (1930; 1931; 1932), as follows:

“Simultaneous white rot fungi: lignin, cellulose and hemicellulose are depolymerised more or less simultaneously (e.g. *Inonotus hispidus*).
Selective white rot fungi: mainly degradation of lignin and hemicellulose, but cellulose is also degraded to some extent (e.g. *Phellinus pini*).”

1.6.1.1 Simultaneous white rot fungal degradation

In simultaneous white rot, the wood cell wall starts degrading by erosion of the lumen surface and progresses towards the middle lamella (Figure 1.18). Holocellulose and lignin are degraded, except the middle lamellae, which is degraded last and only at exposed areas after complete cell wall degradation (Messner *et al.* 2003). Some white rot fungi are not able to degrade the middle lamella, for example *Fomes fomentarius* (Eriksson *et al.* 1990; Messner *et al.* 2003), a phenomenon found and attributed to ascomycetes and some basidiomycetes that degrade hardwood simultaneously (Eriksson *et al.* 1990).

Also, undegraded cell corners can be found far into advanced decay stages (Messner *et al.* 2003). Kirk described that lignin is the physical and chemical barrier to enzymatic depolymerisation of wood polysaccarides (i.e. cellulose) (Kirk 1971; Kirk 1973) and Saka & Thomas (1982) demonstrated that lignin was solubilised from the inner parts of the secondary wall adjacent to the lumen prior to any depolymerisation of cellulose.

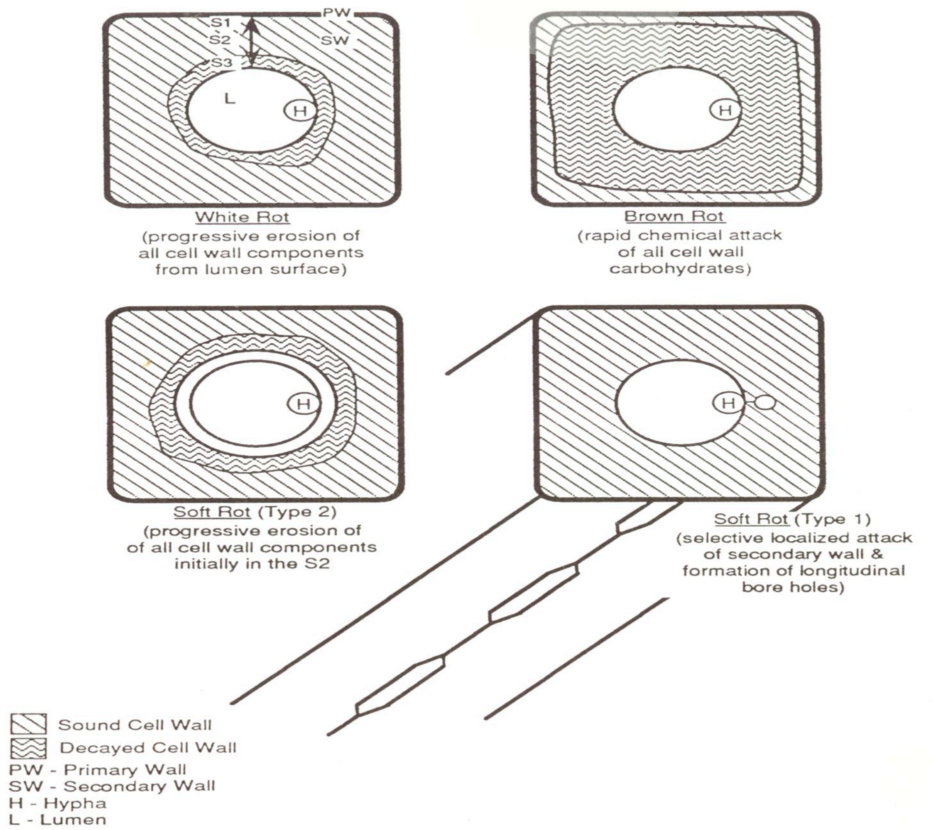


Figure 1.18 Diagram showing the various modes of cell-wall destruction caused by white rot fungi, brown rot fungi, and two types of decay caused by soft rot fungi. Courtesy of Professor J.J. Morrell.

1.6.1.2 Selective white rot fungal degradation

Selective white rot fungi possess the ability to degrade lignin without touching the embedded cell wall cellulose (Blanchette 1984; Eriksson *et al.* 1990). This is a sought after effect especially by the pulp and paper industry, as selective white rot fungi not only have a bleaching effect of the wood pulp for ‘whiter’ paper, they further cause, through their degradative action, a loosening of the cells (Messner *et al.* 2003). Therefore, it is important that delignification occurs in the cell wall as well as in the middle lamella (Srebotnik & Messner 1994). For the polysaccharide hemicellulose, Blanchette (1984) and Eriksson *et al.* (1990) found it to be degraded at the same time as lignin.

Recent research shows that simultaneous, as well as selective, decay can be produced by the same white rot fungus and even in the same piece of wood (Messner *et al.* 2003). ‘Factors that determine different modes of fungal degradation remain obscure’ and unpublished results led Messner *et al.* (2003) to the conclusion ‘that the moisture content of wood is one of the determining factors’.

1.6.2 Brown rot fungi

Of all the wood decay types, brown rot fungi are generally considered the biggest threat for timber in-service, since most framing is manufactured from softwood species.

Brown rot fungi are able to quickly degrade the carbohydrate holocelluloses (cellulose and hemicellulose) of wood early on in the decay process, resulting in a rapid loss of strength (Winandy & Morrell 1993); brown rot is particularly problematic when detected in load bearing structures in a building (Schilling & Jellison 2007).

Holocellulose degradation by brown rot fungi is through extracellular hydrolytic enzymes (Green & Highley 1997; Goodell 2003), which act without the necessity to first remove lignin. The products of hydrolysis are water-dissolved nutrients necessary for fungal metabolism and as an energy supply.

Lignin was for many years thought to be left behind essentially undigested by brown rot fungi but modified through demethylation and oxidation (non-enzymatically), giving brown rotted wood its brown colour (Rypáček 1966; Kirk 1975; Jennings & Lysek 1996; Green & Highley 1997; Goodell 2003). Recent research by Lee *et al.* (2004) showed the ability of the brown rot fungus *Coniophora puteana* not only to depolymerise the cellulosic fraction under traditional brown rot microscopic characteristics but also to form white rot-like erosion and thinning of the cell wall and soft rot-like cavities within the S2 layer

(Figure 1.18). Also of importance, Lee (2004) detected C. *puteana* strains degrading the lignin-rich middle lamellae and producing laccase in liquid cultures. Laccases are phenoloxidases that have a low redox potential, which allow for direct oxidation of phenolic lignin units (Martinez *et al.* 2005), an extracellular enzyme phenomenon previously only known for direct lignin oxidation/degradation by white rot fungi. Hence, Lee *et al.* (2004) suggested that the lignin degrading capacity of brown rot fungi might be greater than previously considered. Another study by Irbe *et al.* (2001) presented data on lignin degradation for three often studied brown rot decay fungi, *C. puteana*, *O. placenta* and *G. trabeum*, degrading 14.7, 11.8 and 25.2% of lignin, respectively. They related their results to the non-enzymatic mechanisms (Fenton reagent's or other powerful oxidants in acidic conditions) thought to be the main reaction involved in lignin degradation by brown rot fungi, as they did not detect extracellular oxidases.

Wood decayed by brown rot fungi appears as a weak amorphous residual skeleton that easily fractures cubically and crumbles when dry. Hartig (1878) was the first to study the progressive stages of depolymerisation by several brown rot fungi in coniferous and deciduous woods. The term he used to describe this type of decay was *red rot*. Hartig observed that the degradation is initiated from the hyphae sitting in the lumen near the cell wall (Figure 1.18) and progresses inward toward the middle lamella.

Since many of the brown rot fungi play only a minor role in lignin modification or demethylation, the type of lignin present in the wood (as described in Section 1.4.1) does not affect the degradation of the cellulose polymers (Nilsson & Daniel 1987). Celluloses and hemicelluloses are cleaved rapidly in early decay stages in softwoods through hydrolytic and galactoglucomannanolytic enzymes. Within the cell walls, the S1 and S2 layers, which are especially rich in polysaccharides, are attacked first before the middle lamellae and the S3 layers are depolymerised (Kirk & Highley 1973). Brown rot fungi additionally can cause decay large distances from their hyphae by releasing diffusible, low molecular weight compounds into the substrate, also known as radical-based systems (Baldrian & Valášková 2008) or iron (Fe)-dependent oxidative chemistry (Schilling & Jellison

2007). These low molecular weight compounds are much smaller in size than enzymes (Baldrian & Valášková 2008) and can therefore penetrate deeper into the substrate matrix (Jellison *et al.* 1991; Filley *et al.* 2002; Goodell *et al.* 2002). Goodell *et al.* (1997) and Goodell (2003) wrote that these compounds are probably quinones produced by brown rot fungi e.g. *Gloeopyllum* sp., *Postia placenta* or *Serpula lacrymans* that are involved in the oxidative redox chemistry (i.e. Fenton reaction). *Gloeophyllum* sp. have been shown to secrete two hydroquinones, 2,5-dimethoxyhydroquinone (2,5-DMHQ) and 4,5-dimethoxycatechol (4,5-DMC) that are able to reduce Fe^{3+} to Fe^{2+} and semiquinone radicals (Kerem *et al.* 1999). The semiquinones can reduce O_2 to •OOH which is a source of H_2O_2 to generate the complete Fenton cycle (Kerem *et al.* 1999).

A recent study by Goodell *et al.* (2006), concerning the iron-reducing capacity of low-molecular-weight compounds produced in wood by brown-, white- and non-decay fungi, revealed that in extracts from wood after colonisation by these fungi, brown rot fungi showed the greatest iron-reducing capabilities, outlining the relevance to the theory for non-enzymatic degradation of wood by brown rot fungi.

The current understanding of degradation of cellulose by basidiomycete fungi was reviewed by Baldrian & Valášková (2008) who found that timber-degrading fungi possess two independent types of systems capable of internal cleavage of cellulose molecules: a) enzyme and b) radical based reactions, with the latter based on redox chemistry by producing hydroxyl radicals that cleave cellulose and hemicellulose non-specifically and also have the ability to modify lignin (Baldrian & Valášková 2008).

1.6.3 Soft rot fungi

The term soft rot was first used by Savory (1954) to characterize decay due to the soft appearance of the wood surface. Today, soft rot fungi are placed within the groups of Ascomycotina and Deuteromycotina (fungi imperfecti). When dry, the decayed wood is brown with cracks and checks that are similar to the appearance of wood affected by brown rot. However microscopically, soft rot fungi produce a different and unique picture of cell wall degradation. Soft rots produce chains of cavities with conical ends within the S2 cell wall layers as shown in Figure 1.18 of softwoods and hardwoods (Daniel 2003). These fungi are very active when conditions are not favourable for brown- or white rot fungi, such as when excessive amounts of moisture are found as in timber with ground contact, in aquatic environments or in cooling towers, or high preservative loadings in timber, are present (Kim & Singh 2000). Additionally, soft rot fungi are often found in the presence of wood- degrading bacteria (Singh *et al.* 1992; Singh & Wakeling 1993; Singh & Wakeling 1996; Singh & Wakeling 1997).

Soft rot fungi do not only produce cavities within the cell wall, they also erode the cell wall, very similar to simultaneous white rot cell wall erosion, beginning from the lumen progressing towards the middle lamella, particularly in hardwoods (Eriksson *et al.* 1990; Eaton & Hale 1993; Daniel 2003). The microscopic evidence of cavity formation and cell wall erosion were referred by Corbett (1965) simply as soft rot types 1 and 2 (Figure 1.18). Type 1 produces cavities within the cell wall, whereas type 2 causes the erosion of all secondary wall layers directly beneath the hyphae. All soft rot fungi normally produce type 1 or a combination of both types, but, contrary to Corbett, Nilsson in 1988 countered that fungi that produce only type 2 should not be defined as soft rots (Nilsson 1988). Cavity formation is very distinct, which makes it reasonably easy to distinguish this decay type from other types of degradation (Zabel *et al.* 1985; Kim & Singh 2000).

In general, the rate and extent of decay by soft rot fungi depends on the wood substrate. Usually, hardwoods are attacked by soft rot fungi to a greater extent than softwoods. This is thought to be related to the higher lignin content and guaiacyl type of lignin in softwoods compared with the lower syringyl- guaiacyl nature of hardwood lignin (Daniel & Nilsson 1997). *P. radiata* is an example of a softwood with high guaiacyl lignin and *Alstonia scholaris, A.* spp. (*Pulai*) are hardwoods with high guaiacyl proportion, providing a high resistance to soft rots (Singh *et al.* 1992).

1.6.4 Bacteria

The degradation of wood cell walls by bacteria was first considered to play a minor role in wood decay compared to brown- or white rot fungi (Wilcox 1968; Liese 1970) until more research was undertaken during the 1980s (Nilsson & Björdal 2008). According to studies of soft rot fungal and bacterial degradation of wood by Daniel & Nilsson (1997), the most common types today are tunneling bacteria (TB), erosion bacteria (EB) and, as reported by Kim & Singh (2000), to a lesser extent, cavitation bacteria and the latter suggested this rare type is closely related to EB occurring only in restricted situations. The main difference between these types, in respect of where they occur in the environment, can be drawn from their oxygen requirements during decay, where EB can tolerate nearly anaerobic or fully anoxic environments while TB are more sensitive to the amount of oxygen present in the wood (Nilsson & Björdal 2008). Hence, in a study by Kim & Singh (1996), it was outlined that EB are mainly responsible for the degradation of waterlogged archaeological wood. In general, wood-degrading bacteria are thought to play a significant role in the degradation of water-saturated wood (Kim & Singh 2000) and are able to decompose the unlignified parts of the cell wall within a few weeks (Schmidt & Liese 1994). Figure 1.19 show aspects of TB and EB degraded *P. radiata* wood cells from observations made by Singh (1989).

However, bacterial decay does not occur solely in waterlogged wood (i.e. wood used as poles in foundations to support buildings in near coastal or river areas (Kretschmar *et al.* 2008)), it was also detected as EB in Copper-Chrome-Arsenate (CCA)-treated *P. radiata* timber, in service for 11 years as a retaining wall (Singh *et al.* 1994), and as EB and TB in CCA-treated *P. radiata* timber from a cooling tower (Singh *et al.* 1992). Latest studies by Kretschmar *et al.* (2008) did focus more on oxygen availability that influences bacterial decay and only older studies, reported in a review by Liese (1992), examined the toxic effects of wood preservatives citing Willeitner *et al.* (1977) that bacterial populations showed detoxification ability of CCA-salt treatments resulting in greater degradation capability of the 'cellar fungus' *C. puteana* on the treated pine sawdust. Schmidt & Liese (1994) attributed this effect to the bacterial population's ability to secrete acids and lower the pH-value of the timber, with the result of former fixed preservative components to be leached out of the timber (similar to oxalic acid production by fungi; Schmidt & Liese 1994). These observations demonstrated that single celled bacteria (mainly bacterial populations) are able to colonise wood and degrade unlignified parts of the cell walls (Daniel 2003) in the presence of wood protecting chemicals (Schmidt & Liese 1994).

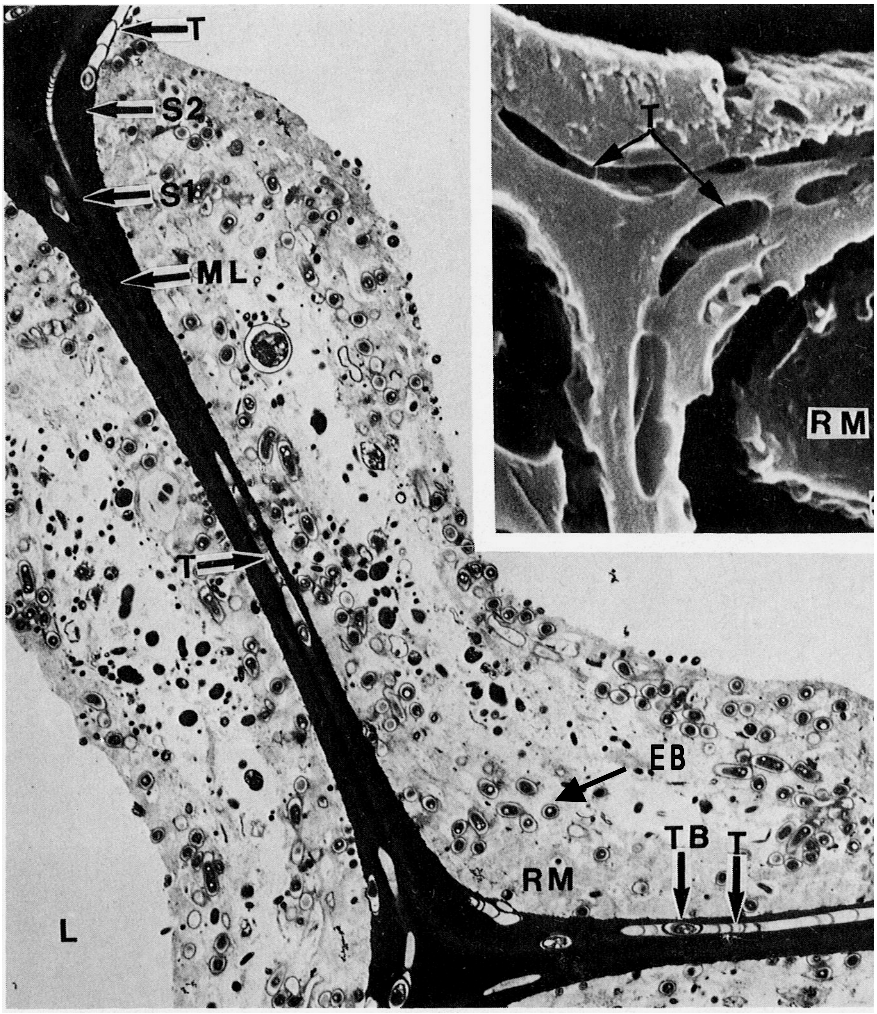


Figure 1.19 Upper right: *P. radiata* corner tracheid region showing prominent tunnels (T) in the wall and residual material (RM); SEM x8,000; Big picture: *P. radiata* transverse section through parts of several tracheids showing the presence of tunneling bacteria (TB) and tunnels (T) in S1 and S2 layers. The residual material (RM), containing mixed bacterial populations including erosion bacteria (EB), has spread into the lumen (L); middle lamella of cells indicated by (ML); TEM x3,700; Courtesy of Dr. Adya P. Singh; originally published by Singh in IAWA Journal Volume 10 (1989).

1.7 Identification of wood decay fungi from construction timbers

Decay fungi can cause a considerable degree of destruction to wood in buildings, often making expensive remediation procedures a necessity to bring the building back into a habitable state. In Europe and North America, and as shown for the first time with this study in New Zealand, most damage to construction timber is attributed to brown-rot decay fungi, which are capable of degrading the carbohydrates (cellulose and hemicellulose) in coniferous woods used in buildings (Schmidt 2007). New Zealand has no regulation that makes it compulsory to identify fungi occurring in buildings; hence, in all remediation cases, the questions are as follows: how far the decay type(s) advanced in the construction and how many framing members and other building components need to be replaced? Nevertheless, fungal species identification is critical for a successful remedial treatment of the damage as well as for scientific purposes, since different species will cause different kinds of damage requiring different kinds of actions (Högberg & Land 2004; Schmidt & Kallow 2005). One aspect of this PhD thesis research concerned choosing the correct remediative treatment; methodology and results addressing this aspect are presented in Chapter 3 through data of mass loss caused in preservative-protected timber for some brown-rot decay fungi isolated from New Zealand buildings, outlining their tolerances and susceptibilities towards certain chemicals.

To detect and identify decay fungi from construction timber, there is no single optimal method available (Jellison & Jasalavich 2000). Currently, the combination of traditional methods based on morphological characteristics of fruiting bodies, mycelium and decay patterns in wood (Högberg & Land 2004) and molecular ‘forensic’ methods such as the ***Polymerase Chain Reaction*** (PCR) and sequencing (Schmidt 2007) are used for identification purposes. Other techniques for identification and detection used in the past, all with limited degrees of success, include culturing, chemical fluorescence staining, sonic analysis, Nuclear magnetic resonance (NMR), electrical resistance and serological methods (Goodell & Jellison 1988).

In this PhD thesis research, culturing and mycological examinations were conducted together with the use of the molecular PCR technique, to identify decay fungi from leaky New Zealand buildings and their principles are discussed in this section.

1.7.1 Mycological examinations

To identify a fungus, traditionally morphological examinations are carried out and are understood as macro- and microscopic observations and recorded to categorise the causal fungus. Either fungal fruiting bodies, strands on building material, as mentioned in Section 1.3, or pure cultures can be examined to aid in identification. Often straight forward and as the preferential method for identification are the macroscopic appearance of a fruiting body and the microscopic analyses and measurement of their reproductive spores, using keys or illustrated books (Cunningham 1965; Ryvarden 1978; Grosser 1985; Breitenbach & Kränzlin 1986; Ryvarden & Gilbertson 1993; 1994; Bravery *et al.* 2003; Huckfeldt & Schmidt 2006a). Today, a diagnostic key in respect of fruiting body formation for fungi growing on structural timber can be found on the Internet (Huckfeldt 2002). However, one cannot solely rely on morphological examinations of fruiting bodies, as the growth environment for decay fungi does not necessarily allow fruiting body formation. If they are present, they are often represented in a very unnatural shape, sometimes referred to as a "dark" fruiting body (Huckfeldt & Schmidt 2006a).

A second classical identification of decay fungi from buildings is via their strands, which some may produce in this environment. Falck (1912) was the first to establish a key for wood decay fungi in buildings based on their strands, which was later reviewed and modified by Bavendamm (1936), Bravery *et al.* (2003) and Huckfeldt & Schmidt (2006b). About 60 indoor wood decay fungi found in Europe have been recently described and illustrated in the literature (Huckfeldt & Schmidt 2006a). Recently, a new identification key for strand-forming indoor wood decay fungi, adding new fungal species to the original work of Falck (1912), has been published (Huckfeldt & Schmidt 2006b). This key describes 20 common European fungal species including brown- and white-rots (Schmidt

2006), based on observations made on mycelia and strands from affected buildings and on wood samples that have been incubated with pure cultures in the laboratory. All species described in the key were confirmed and identified by traditional pure culture examinations combined with rDNA-ITS sequencing (Section 1.7.2) earlier by Schmidt & Moreth (2002; 2003) to avoid misidentification.

Pure fungal culture examinations are the third traditional means for identification purposes. Extensive work upon mycelium formation and growth on agar medium under defined laboratory conditions has been done in the past, which led to the establishment of many identification keys for wood decay fungi. Important keys commonly used following microscopic characteristics of the growing fungal hyphae were published by Nobles (1965), von Arx (1967), Stalpers (1978), Rayner & Boddy (1988) and Lombard & Chamuris (1990). However, there are some limitations to identification keys for fungal mycelia as they are unable to differentiate between closely related fungi such as the various *Coniophora* (Schmidt *et al.* 2002) and *Antrodia* (Schmidt & Moreth 2003) species. To overcome these limitations in fungal species identification, molecular techniques based on objective information provided through fungal specific deoxyribonucleic acid sequences (DNA) are called upon for species confirmation.

1.7.2 Molecular techniques

Molecular techniques enable the identification, characterisation, classification and quantification of organisms and have been applied for building related fungal decay studies since the 1980s (Palfreyman *et al.* 1988; Jellison & Goodell 1988; Schmidt & Kebernik 1989). Most notably, methods depending on protein analysis (SDS-PAGE), antibody binding (Immunotechnology) or DNA sequence patterns (PCR based techniques) have been used to analyse the organisms.

1.7.2.1 Protein-based techniques

Some specific proteins are separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and can be recognised by their apparent molecular size; this is a useful tool to discriminate organisms at the species level (Palfreyman 1998) and has been used to distinguish wood decay fungi (Schmidt & Moreth 1995). Closely related species such as the two fungi *S. lacrymans* and *S. himantioides* were separated by their respective specific protein fingerprints, i.e. banding pattern (Schmidt & Kebernik 1989) or to confirm phylogenetic relationships between different tree pathogens of *Heterobasidion annosum* (Karlsson & Stenlid 1991). However, in terms of building decay fungi identification, this approach is regarded to be of limited value, as it requires the time consuming isolation and establishment of pure fungal cultures (Schmidt 2006).

The identification of indoor wood decay fungi has also been achieved with immunological (serological) methods. With these techniques, either polyclonal (*in vivo*) or monoclonal (*in vitro*) antibodies are used (Palfreyman 1998) to detect and identify decay fungi in wood and pure culture with the aid of systems known as 'Western' blotting, immunofluorescence or an enzyme-linked immunosorbent assay (ELISA) (Clausen 2003). Potential limitations in these systems have been identified as cross-reactions with non-target organisms even if monoclonal antibodies are used (Schmidt 2007). Nevertheless, the diagnostic potential lies in the identification and early detection of wood decay fungi, excluding fungal isolation and purification steps, with the possible prevention of massive wood degradation in buildings (Clausen & Kartal 2003). As yet, the potential of antibody systems for an on-site screening device for common building decay fungi hasn't been realised (Schmidt & Huckfeldt 2006a).

1.7.2.2 DNA-based techniques

DNA-based methods involve the application of the already mentioned PCR technique, using universal or even taxon-specific primers (Gardes & Bruns 1993; Martin & Rygiewicz 2005). Primers are short, synthetically produced DNA molecules binding to target DNA at the beginning and end of a particular DNA sequence of interest. Using DNA polymerase and a strict temperature regime, the target DNA is amplified (Mitchell & Zuccaro 2006; Schmidt 2006). Amplification in every cycle is achieved by:

- **Denaturation**: melting the DNA double helix into single strands at high temperatures between 94-98°C followed by
- **Annealing**: where the temperature is lowered to 50-65°C to allow the two oligonucleotide primers to hybridise to the complimentary nucleic acid region of the single stranded DNA template.
- **Extension**: where the temperature rises again to 72°C to the optimum polymerisation (working) temperature of the heat resistant polymerase used to copy (extend) the target DNA (synthesis of a complimentary strand). The extension, which started at the primer sites, is consolidated through the enzyme adding one of the four deoxynucleotides (dATP, dGTP, dCTP and dTTP) (Mullis 1994) in the order in which they can pair, thus synthesising the opposite strand of the DNA.
- Following the extension phase, cycle is repeated and the temperature is again raised to approximately 94°C to melt the strands apart, thus providing template DNA for the primers to anneal to.
- Every time a PCR cycle is completed, the amount of PCR product is doubled, therefore after 20 cycles, more than a million copies of the template DNA have been synthesized.

Until late 1990s, few reports concerning the identification of indoor wood decay basidiomycetes were published using molecular DNA based techniques (Moreth & Schmidt 2000). A common indoor basidiomycete fungus, *S. lacrymans* and close relatives such as *S. himantioides,* were studied by exploring the systematic feasibility of randomly amplified polymorphic DNA by the polymerase chain reaction (RAPD-PCR). (Palfreyman *et al.* 1995; Theodore *et al.* 1995; Schmidt & Moreth 1998; Moreth & Schmidt 2000). It was shown that RAPD-PCR could easily and rapidly differentiate between organisms without any prior knowledge of specific DNA sequence information (Schmidt & Moreth 1998) using one short and randomly chosen primer. Differentiation results of fungal PCR products were made visible in ethidium bromide stained agarose gels, distinguishing between different/same organisms through banding patterns according to the presence or absence of bands (polymorphism). Though the technique is fast when starting from pure cultures (Schmidt 2006), RAPD analyses need axenically grown cultures, as the short primers are very sensitive to contamination (Schmidt &

Moreth 1999). However, RAPD is valuable in discriminating specific fungal species like *S. lacrymans* at the taxonomical level but is thought unsuitable for the identification of unknown samples by comparison, as similar banding patterns might exist by chance in yet unexplored fungi (Schmidt 2006).

A second DNA-based analyses technique, known as Terminal Restricted Fragment Length Polymorphism (T-RFLP) based on ribosomal DNA (rDNA) has been used successfully for the study of bacterial communities (Blackwood *et al.* 2003), ectomycorrhizal hyphae diversity in soil (Dickie *et al.* 2002) and to detect, discriminate, identify and categorise species of wood decay fungi (Zaremski *et al.* 1998; Diehl *et al.* 2004; Råberg *et al.* 2004, 2005, 2006). This analysing technique combines three independent molecular methods, starting with a PCR reaction with the inclusion of fluorescent labelled primers, followed by restricted fragment length polymorphism (RFLP), which utilises digestion with selected restriction enzymes and nucleic acid electrophoresis (Råberg *et al.* 2004) giving the length of the terminal fragment(s) in a digital high-resolution output (Lord *et al.* 2002). The technique allows screening of a community of wood decay fungi from one sample source at a time, with each fungus represented by a peak (fragment size profiling) in the electrophoresis gel (Råberg *et al.* 2004). T-RFLP is an often used technique as it has the advantage of being fast and inexpensive (Schmidt 2007) and is a favoured database for the identification of wood-decay and associated fungi (Diehl *et al.* 2004; Råberg *et al.* 2005). However, it is possible to have a false positive result as yet unanalysed species might be represented with the same fragment size (Schmidt 2007).

The DNA-based molecular method used in this PhD thesis research, which avoids the main limitations of the techniques previously described in this section, is the combination of rDNA-PCR and sequencing of the Internal Transcribed Spacer (ITS) region (Section 1.7.2.3) of the target organism.

1.7.2.3 Sequencing of ribosomal DNA

The 'copy machine' PCR has enabled investigations of the ribosomal DNA (rDNA), the unique genetic fingerprint of an organism (plant, animal, human, fungus), to be identified, categorised and placed into family relations (phylogenetic studies). Within an organism, repetitive units of the nuclear ribosomal DNA are present, consisting in Eukaryotic fungi of the conserved coding domains 18S, 5.8S, 28S and 5S rDNA, ribosomal genes that vary little in their nucleotide sequences (i.e. A, T, C, G) and are often used for phylogenetic studies of genera, families and orders (Schmidt 2006). Further, these conserved domains are interspersed by non-coding variable spacers called ITS I between 18S and 5.8S, and ITS II between 5.8S and 28S. This ITS region is species specific and can show differentiations in form of evolutionary mutations, hence preferentially used for species identification (Högberg & Land 2004). Additionally, an intergenic spacer (IGS) is located between the 28S and 18S of the next rDNA unit, sometimes divided into IGS I and IGS II, if a 5S rRNA gene is present in-between (Huckfeldt & Schmidt 2006a). A diagram of a full rDNA unit, including the restriction sites for some chosen primers, is presented in Figure 1.20.

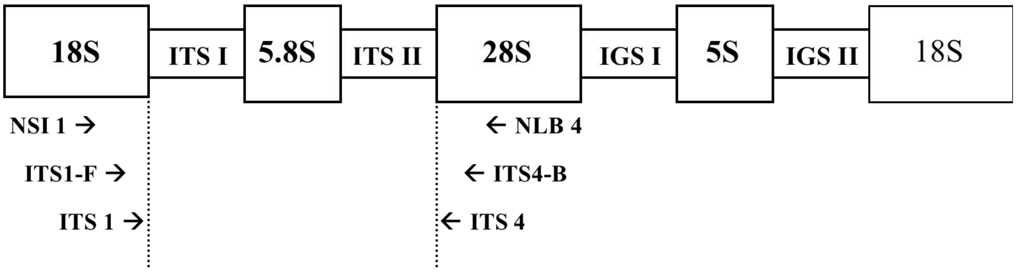


Figure 1.20 Schematic diagram of one rDNA unit. Forward primer NSI1, ITS1-F, ITS1 and reverse primer NLB4, ITS4-B, ITS4, positioned below rRNA genes 18S and 28S, showing the part of the rDNA spectra these primer cover.

rDNA-ITS sequencing is the most important current tool in molecular analyses, with many data sets available to compare house-rot fungi (Schmidt & Moreth 2002, 2003; Jellison *et al.* 2003; Moreth & Schmidt 2005).

Amplification of the ITS-regions (Figure 1.20) can be achieved with, but is not limited to, the following primers:

- **ITS 1 to 4**, universal primer pairs (White *et al.* 1990),
- **ITS1-F** and **ITS4-B**, fungal specific primer pair (Gardes & Bruns 1993),
- **NLB4** and **NSI1**, fungal specific primer pair (Martin & Rygiewicz 2005).

The first full ITS sequence of *Serpula lacrymans* was made available in 1999 by Schmidt & Moreth (1999), initially using the forward primer ITS1 and reverse primer ITS4 developed by White *et al.* (1990) for its identification. Further, out of the ITS II base sequence obtained, they developed taxon specific primers for *S. lacrymans* and used the same technique for many other wood-decay fungi from buildings (Moreth & Schmidt 2005).

As of late 2008, many rDNA-ITS sequences of wood-decay fungi have been sequenced, making it a powerful tool to identify unknown fungal samples through sequence comparison (Schmidt 2007) using the *Basic Local Alignment Search Tool* (BLAST) on either one of these publicly available international GenBank online databases:

- **EMBL**: European Molecular Biology Laboratory: www.ebi.ac.uk/embl
- **NCBI**: American GenBank: www.ncbi.nlm.nih.gov/Genbank
- **DDBJ**: DNA Data Bank of Japan: www.ddbj.nig.ac.jp

The online BLAST function provides researchers a method for rapid searching and comparing all internationally available nucleotide databases (Altschul *et al.* 1990) for fungal taxation and identification. However, one needs to be aware that the potential risk of using BLAST lies in being an open source, and depositions of sequences might have been made under incorrect species names (Högberg & Land 2004).

1.8 Timber treatments

The natural resource, wood, can be found in many applications, either as roundwood in products such as telephone and electric power transmission poles, log homes, marine piers and in city foundations where high ground water levels persist (e.g. Amsterdam, Eberswalde), or in its processed form, timber (lumber), such as framing, decks, fences and retaining walls. As outlined in Section 1.4, most timber products in use, particularly those cut from plantation softwoods, have a limited or very low natural durability if not kept dry during their service lives. Particularly, insect organisms e.g. wasps, beetles, termites, marine borer (e.g. *Teredo navalis*), bacteria and wood decay fungi can be a threat to unprotected timber, thus making its protection against these organisms a necessity to enhance its lifespan.

Timber protection can be seen as the sum of all measures to prevent timber degradation, caused either through fungal and animal organisms, or through physical effects of the environment (weathering), to achieve a long-lasting preservation of the product. These timber-protecting measures can be divided into four main strategies:

- **Use of naturally durable wood species**: choosing the right timber for the intended application;
- **Constructional and design feature considerations**: to avoid moisture accumulation and keeping the timber dry; planer gauged timber to reduce insect attack;
- **Surface coatings**: to protect the timber from climatic influences;
- **Timber treatments**: using biocides to protect the timber from wood degrading organisms.

Although intended only to be harmful to the target organism, the use of timber treatments (biocides) can have an adverse effect on the environment (leaching out of the preservative, see Figure 1.21) and may have real or suspected harmful effects on non-target plants, animals and/or humans. However, in certain applications such as structural timbers with a high-risk moisture exposure (e.g.

posts embedded in the ground supporting balconies), wood preservatives are required to guarantee the intended performance. These timber treatments can be fungicides or insecticides, or, commonly, a combination of both.

Figure 1.21 Greenish leachate staining the concrete in front of a CCA-treated pole.

This section on timber treatments presents commonly used wood preservatives, and their application in New Zealand constructions and buildings, and their biochemical mode of action.

1.8.1 Wood preservatives in use to protect New Zealand's buildings

Initial considerations in terms of timber preservation, for the product to be fit for purpose, haven't changed since Pliny the Elder (27-79AD), an early Roman naturalist, advocated the use of oil of cedar (*cedri oleo peruncta*) to protect timber against decay (Ulrich 2007) and are therefore important and include the following questions:

- What is the degree of protection required?
- What are the hazard(s) against which protection is desired?
- What is the available information about the use-intended toxicities of the biocide?

For a long time, New Zealand's principal wood-destroying organisms in houses were thought to be insects, particularly dominant the common house borer *Anobium punctatum* De Geer (Carr 1957), with fungal decay not being considered as a serious hazard. A statement made by Carr in 1957 addressed the requirements of timber preservation in constructions in New Zealand and should be still true today, fifty-one years later: "In practice, decay may be caused by inadequate ventilation, guttering or plumbing leakages, condensation or poor design, but these are exceptions rather than the rule in modern construction". However, as discussed in Section 1.2, it is not, hence various timber treatments find their application in house constructions.

In early 2004, in a response to the weathertightness problems of some buildings constructed during the 1990s and early 2000s, Clause B2 (Durability) of the New Zealand Building Code (NZBC 2004) and the Standards NZS 3602:2003 *Timber and wood-based products for use in buildings* and NZS 3640:2003 *Chemical preservation of round sawn timber* were released as revised documents, setting new minimum levels of timber treatment for selected parts of every new building. Common treatments used to protect timber in New Zealand since the Timber Preservation Authority was established under the Timber Preservation Regulations Act 1955 have been boron and CCA formulations. Since the late 1980s, light organic solvent preservatives (LOSPs) became introduced (Hedley & Page 2003). Nowadays, alternatives to CCA in residential applications are available as there are concerns with this preservative, including public perception of possible arsenic exposure, leaching of the metal oxides and poor recycling or other end-of-life disposal options. (Green & Schultz 2003). However, in New Zealand, CCA is still the only approved treatment for use in marine situations. Alternative treatments for all other applications exist and are sometimes referred to as "second-generation" wood preservatives and include copper-organic mixtures such as alkaline copper quaternary (ACQ) and copper azole mixtures (CuAz) (Green & Schultz 2003). A full list of all available timber preservatives used in various New Zealand applications is provided in Table 1.4, alongside their respective identification codes which are included in the treatment identification brand treated timber products.

Table 1.4 Available timber treatments in New Zealand and their respective identification codes. According to NZS 3640:2003 Chemical preservation of round sawn timber, New Zealand Standard (2003).

Preservative	Full name	Code
CCA oxide	Copper chrome arsenate oxide	01
CCA salt	Copper chrome arsenate salt	02
Boron	-	11
TBTO	Bis-(tri-n-butyltin) oxide	56
CuN	Copper naphthenate	57
CuAz	Copper azole	58
TBTN	Bis-(tri-n-butyltin) naphthenate	62
IPBC	3-Iodo-2-propynyl-butyl-carbamate	63
Propiconazole + Tebuconazole	-	64
Permethrin	-	70
ACQ	Alkaline copper quaternary	90

1.8.2 Hazard classes for framing and other construction timbers

Hazard classes for wood preservatives have been defined to guide the designer, specifier or consumer in selecting the right level (retention), type (water or LOSP based) and kind (Boron, IPBC etc.) of treatment best suited for the intended application. These guidelines/regulations are found in the New Zealand Standard NZS 3640:2003 *Chemical Preservation of Round Sawn Timber* and are applicable to every new building using *P. radiata* framing timber. Table 1.5 identifies the six different hazard classes usually present at a building site, common preservatives used within these hazard classes and the type of protection offered for typical end-uses. Table 1.5 excludes H6, a hazard class relevant only to constructions in the marine environment such as wharf and marina piles immersed in seawater or estuarine ground.

Treatment of framing timber is specified to preservative retention levels in either of two Hazard Classes, H1.2 or H3.1, depending on the location within a building and the perceived risk of inadvertent wetting. There are a number of preservative formulation options, most of which are naturally colourless. Therefore, as an aid to identification on site, colour coding was introduced and implemented by adding a dye or stain to the preservative treatment solution. For example, H1.2 LOSP treatment is coloured blue, but H3.1 LOSP treatment is coloured green.

Table 1.5 Hazard classifications for timber in constructions after NZS 3640:2003.

Hazard class	Exposure	Preservatives	Retention (% m/m)*	Biological hazard	Typical end-uses
H1.1	Protected from the weather, above ground	*Waterborne*		Borer	Interior finishing timber
		Boron	0.10-0.20		
		CCA	0.04		
		LOSP			
		Permethrin	0.0060		
		Cypermethrin	0.0060		
		Deltamethrin	0.0006		
H1.2	Protected from the weather, above ground, but with a possibility of exposure to moisture	*Waterborne*		Borer, decay	Wall framing
		Boron	0.40		
		LOSP			
		TBTO	0.06		
		TBTN	0.06		
		IPBC +	0.025 +		
		Permethrin	0.006		
H3.1	Exposed to the weather, above ground	*Waterborne*		Decay fungi and borer	Cladding, facia, joinery
		Boron	0.80		
		CCA	0.37		
		ACQ	0.35		
		CuAz	0.2288		
		LOSP			
		TBTO	0.08		
		TBTN	0.08		
		CuN	0.05		
		Propiconazole + Tebuconazole	0.06		
H3.2	Exposed to the weather, above ground or protected from the weather but with a risk of moisture entrapment	*Waterborne*		Decay fungi and borer	All H3.1 uses, plus structural and decking, pergolas, external beams, posts not in ground
		CCA	0.37		
		ACQ	0.35		
		CuAz	0.2288		
		LOSP			
		CuN	0.10		
H4	Exposed to the weather, in ground or fresh water	*Waterborne*		Decay fungi and borer	Fence posts, landscaping timbers
		CCA	0.72		
		ACQ	1.02		
		CuAz	0.46		
H5	Exposed to the weather, in ground or in fresh water	*Waterborne*		Decay fungi and borer	House piles and poles, posts in ground for decks, verandas, pergolas
		CCA	0.95		
		ACQ	1.35		

Preservatives highlighted in green within a specific hazard class have been used in this PhD research work.

* Retentions are based on the oven dry weight of wood, here presented indicative only. Full requirements for each hazard class and respective limitations to the use of all preservatives are contained in NSZ 3640:2003 Chemical preservation of round and sawn timber, and Amendments No.3 (August 2006) and No.4 (March 2007).

1.8.3 Biochemical mode of action of wood preservatives - principles

The approach currently used to preserve timber products in service from degradation by insects or decay fungi is impregnation with biocidal compounds designed to inhibit the activity and viability of these organisms. These can either be fungicides or insecticides, or usually a combination of both. Broad- spectrum insecticides like permethrin and cypermethrin are examples of the most common active ingredients of modern LOSP treatments when control of wood-boring insects or termites is required. They are synthetic neurotoxin chemicals belonging to the family of pyrethroids, which act for example by blocking sodium channels in neurons, leading to paralyses and death of the insects (Eaton & Hale 1993).

Wood preservative formulations may have multiple active ingredients incorporated, each having its own mode of action, most of which are still unknown or unreported. Furthermore, non-active ingredients such as ethanolamine in some second-generation copper-based preservative solutions, and which act as a fixative for copper (Zhang & Kamden 2000), may even stimulate fungal growth as it contributes significant amounts of nitrogen to the wood (Humar & Lesar 2008). Hence, the synergistic or antagonistic mechanisms between different ingredients in a treatment solution are an important factor, determining the effectiveness of the treatment in its end-use situation.

Many fungicides prevent normal cellular activity by inactivation of enzymes, disruption of cell membranes or inhibition of protein synthesis (Eaton & Hale 1993). More specifically, some actives such as polyene and triazole fungicides (including tebuconazole used in copper-azole preservative solutions and tested in this study), act on fungal ergosterol (Robson 1999). The active ingredient copper in preservative formulations is the oldest and without doubt the most broadly effective treatment and has been used for more than 200 years (Humar & Lesar 2008). Even though copper on its own has the disadvantage of not fixing to the wood this was overcome by adding chromium to the solution, resulting in CCA, the most often used preservative for residential constructions worldwide during the last half of the twentieth century (Schultz *et al.* 2007). The arsenic compound in the formulation further enhanced resistance against insects and copper-tolerant fungi (Richardson 1997) like *Antrodia vaillantii*. This fungus is an example of the problems facing the right choice of preservative, as *A. vaillantii* excretes high amounts of oxalic acid (Schmidt 2006), which is believed to detoxify copper by forming insoluble copper oxalate (Green & Clausen 2005). Subsequently, copper-tolerant fungi have been used to bio-recycle CCA treated wood (Humar *et al.* 2004; Kartal *et al.* 2004). In terms of leaky buildings, considerations have to be made in respect to the right choice of remedial treatment prior to any framing replacement; as, for example, the findings of this PhD thesis research showed that the decay fungus might be copper tolerant as described in Chapter 3.

Therefore, second-generation copper preservatives often have secondary fungicides added such as azoles (tebuconazole and propiconazole) as in copper azole or quaternary ammonium compounds such as didecyl dimethyl ammonium chloride (DDAC) as in Alakaline copper quaternary treatments. Having a long history as wood preservatives, borates have an extremely low mammalian toxicity with a broad range of activity against fungi and insects (Schultz *et al.* 2007). The mechanism behind DDAC fungicide is believed to be a disruption of intermolecular interactions, causing dissociation of cellular membrane bilayers, which compromises cellular permeability controls and induces leakage of cellular contents (Siegel & Sisler 1977). The organic biocides in the family of triazoles, including propiconazole and tebuconazole, originated as broad-spectrum agrochemical preservatives to treat plant pathogenic fungi. They are highly effective against a broad range of basidiomycete fungi and they exhibit good stability and leach resistance in wood (Schultz *et al.* 2007).

Another way to limit leaching and/or retaining of non-fixing biocide actives in the wood is by adding hydrophobic additives to the treating solution, such as oils, waxes, paraffins and silicones (Humar *et al.* 2005a; Schmidt 2006). Rapp *et al.* (2005) used vegetable oil, which is deposited in the wood cell lumina and subsequently reduces the water uptake capacity.

1.9 Hypothesis, Aims and Objectives of PhD thesis research

The Ph.D. thesis research concerned the New Zealand so-called leaky buildings crisis, which developed in the late 20th and early 21st Centuries. One major unknown factor was the identity of the causal agents and if fungal, as suspected, the variability of decay organisms throughout the buildings affected. Further problems associated with the presence of decay fungi within leaky buildings included loss of property value and possible health and safety risks to the owners, loss of confidence in the value of wood framing as a building material and associated spin off effects on the wood product and building industry. This thesis research aimed to establish data to contribute and assist in the understanding and subsequent remediation process of leaky buildings by testing the following:

1.9.1 Hypotheses

- Examination of degraded framing timbers of leaky New Zealand buildings will reveal that there is more than one causative decay fungal organism.
- Decay fungal isolates will be susceptible to wood preservatives accredited for the use of framing timber in New Zealand.

1.9.2 Aims

The primary aims of this research were

i. to identify the causative agents of fungal decay in leaky buildings;
ii. to identify optimal preservative systems for protection of framing against wood decay fungi in New Zealand;
iii. to increase understanding of their ecology with particular reference to an aerial spore study; and
iv. to increase understanding of micromorphological changes in untreated *Pinus radiata* framing timber.

1.9.3 Objectives

The primary objectives for this thesis were five-fold:

1. Obtain decayed leaky building samples to isolate causative fungi on artificial growth media and produce pure cultures.
2. Identify decay fungi by either
    a. extraction of fungal DNA from isolated pure cultures followed by applying the polymerase chain reaction (PCR) to obtain amplicons of regions of ribosomal DNA (rDNA), which were subsequently sequenced, or
    b. by morphological examination of fruiting bodies;
3. Use selected key decay fungi to test the minimum inhibitory concentration (MIC) of commonly used framing timber treatments in New Zealand to determine decay potential;
4. Assess aerial fungal spores to identify and compare amounts of (basidio-) spores of internal air, wall cavity air and exterior air at leaky buildings;
5. Investigate micromorphological changes in *P. radiata* decay fungal colonised timber with correlating light microscopy (LM) and Field-emission scanning electron microscopy (FE-SEM).

1.10 Preamble

This thesis comprises six chapters. Chapter 1 gives the introduction of the research topic and reviews the literature. Chapters 2 - 5 are experimental chapters, each presenting its own Materials and Methods, Results and Discussion sections. The research and results as described in parts of Chapters 2, 3 and 4, respectively, Isolation and Identification, Mass loss in treated and un-treated timber and Aerial spore studies, were prepared, orally presented and published by the candidate under the guidance of his supervisory panel and co-authors Professor Roberta Farrell, Dr. Robin Wakeling and Dr. Mick Hedley, in two papers to the *International Research Group on Wood Protection* annual conferences in Jackson Lake, Wyoming, USA, 2007 and in Istanbul, Turkey, 2008. The documents are IRG/WP/07-10620 and IRG/WP/08-10649 and are given in Appendix 1. Chapter 6 unites the research of the thesis in a concluding discussion.

2 Isolation and identification of wood decay fungi

2.1 Introduction

Identification of decay fungi from New Zealand leaky buildings was the first objective of the PhD thesis research. It was accomplished by morphological examination of fruiting bodies at leaky building sites or extraction of fungal DNA from isolated pure cultures from leaky building sites, followed by applying the polymerase chain reaction (PCR) to obtain amplicons of regions of ribosomal DNA, which were subsequently sequenced. The latter is the preferred method of identification of the 21st Century as it holds less determination error than traditional microscopy (Horisawa *et al.* 2004; Schmidt & Moreth 2003).

The main reason for difficulty with morphological examination of fruiting bodies is the building situation itself where often no fungal fruiting bodies were found. Alternatively, fruiting bodies were represented as a so called “dark” fruiting body having an unnatural shape; Huckfeld & Schmidt (2006a) estimated that about 15% of these “dark” fruiting bodies are not correctly identified. Therefore, sequencing is more practical and not subjective, and it minimises human error albeit, the identification of DNA, which relies on only a small part of the genome for identification of an organism, can sometimes be problematic.

2.2 Materials and Methods

2.2.1 Sourcing leaky building wood samples

Four hundred and twenty one framing and other moisture-compromised building material samples were selected from a much larger sample group derived from predominantly exterior wall cavities of approximately 2000 leaky buildings throughout New Zealand. The samples were made available by Beagle Consultancy Ltd, Rotorua, New Zealand, a company specialised in the examination of deteriorated building materials.

2.2.2 Media used

The following three media and reagents were used throughout this PhD thesis research; all were made to 1 litre with distilled water and autoclaved for 20 minutes at 120°C before cooling and subsequently pouring agar plates with 15-20 ml added to 100 mm Petri plates:

- Malt Agar **(MA)** – 1.5% malt extract (DANISCO Ltd., Manukau City, Auckland, New Zealand), 2% agar (DANISCO Ltd., Manukau City, Auckland, New Zealand).

- Basidiomycete semi-selective agar **(BMA)** (Worrall 1991) – 1.5% malt extract, 0.2% yeast extract (OXOID Ltd., Basingstoke, Hampshire, England), 1.8% agar, 0.2 g/l chloramphenicol (Sigma-Aldrich Inc., St. Louis, USA), 0.06 g/l benlate, 0.1 g/l streptomycin sulphate (Sigma-Aldrich Inc., St. Louis, USA). 2 ml/l lactic acid (APS Ajax Finechem, Australia) were added to the autoclaved medium once cooled to at least 50°C.

- Acidic malt extract agar **(AMA)** – 1.5% malt extract, 2% agar. 2 ml/l lactic acid were added to the autoclaved medium once cooled to at least 50°C.

2.2.3 Isolation protocol

In the laboratory, wood samples were cut to approximately 3 x 30 mm size using sterile chisels and razor blades, before transfer to laminar flow cupboard where they were surface sterilised by immersion in 70% ethanol, flamed (for approximately 10 seconds), and then placed on 2% MA, BMA or AMA media at room temperature ranges of 20 to 24°C. Pure cultures were then subsequently established from cultures growing out from the wood samples by inoculation of hyphal tips.

2.2.4 Storage

Long term storage of pure cultures, to ensure later use of the identified fungal strains for further studies such as the wood decay test as described in Chapter 3, was achieved as follows:

1. Cultures were allowed to grow on 1.5% MA Petri dishes for two to three weeks at laboratory room temperature, approximately 20 degrees Centigrade (C).
2. Using autoclaved transfer pipettes, from each culture, 7 to 8 small segments of the fungus on the agar medium, about 1 x 0.5 x 0.3 cm, were placed into 1.5ml sterile plastic vials, filled with either distilled water or 10% glycerol in water and closed with a screw top.
3. Labelled vials were subsequently stored in a refrigerator (for the samples in distilled water) or in a Minus 40 ° C freezer for glycerol immersed cultures.

2.2.5 Identification from fruiting bodies

All fruiting bodies received or collected from leaky buildings were morphologically assessed by their macroscopic appearance and measurement of reproductive spores using the following keys and illustrated literature books:

- ***Fungi of Switzerland Vol. 2, non-gilled fungi.*** Breitenbach, J. & Kränzlin, F. (1986) Verlag Mykologia, 2nd Edition, Lucerne, 416 pp.
- ***Polyporaceae of New Zealand.*** Cunningham, G.H. (1965) New Zealand Department of Scientific and Industrial Research bulletin, **164**: 304 pp.
- ***Pflanzliche und tierische Bau- und Werkholz-Schädlinge.*** Grosser, D. (1985) DRW Verlag Weinbrenner-KG, Germany.
- ***Hausfäule Pilze – Hausschwamm, Kellerschwamm, Porenschwamm.*** Huckfeldt, T. (2002) www.hausschwamminfo.de

- ***Hausfäule- und Bauholzpilze. Diagnose und Sanierung*****.** Huckfeldt, T. & Schmidt, O. (2006a) Verlagsgesellschaft Rudolf Müller, Köln 2006, 377 pp.
- ***The Polyporaceae of North Europe*** **Vol.1.** Ryvarden, L. (1978) Oslo.
- ***European Polypores*****. Part 1.** Ryvarden, L. & Gilbertson, R.L. (1993) Synopsis Fungorum 6, Fungiflora Oslo.
- ***European Polypores*****. Part 2.** Ryvarden, L. & Gilbertson, R.L. (1994) Synopsis Fungorum 7, Fungiflora Oslo.

For the avoidance of doubt and to minimise the risk of misidentification, a second independent analyses was carried out for each fruiting body by mycologist and forest pathologist Ian Hood, Scion, Department of Forest Protection and Biosecurity, Rotorua, New Zealand.

2.2.6 Identification from pure cultures

2.2.6.1 Preparation of Polymerase Chain Reaction suitable DNA samples

Fungal cultures were grown on 2% MA at 26°C for 12 days. For easier collection of mycelium, an autoclaved cellophane jam jar preserve cover was placed onto the media surface before the plate was inoculated with the fungus.

A modified 2X cetyltrimethylammonium bromide (CTAB) lysis buffer protocol (Vogler & Bruns 1998) was used for extracting DNA from fungal cultures. Mycelium was collected in 1.5ml tubes, suspended in 300µl 2X CTAB buffer (100-mM Tris HCl, pH 8.0, 1.4 M NaCl, 20 mM Ethylenediaminetetraacetic acid (EDTA), 2% CTAB, 0.2% ß-mercaptoethanol), ground with a plastic pestle and incubated at 65°C for 1 hour, extracted with an equal volume of chloroform, and centrifuged at 10,000 x *g* for 30 min at room temperature. The upper aqueous phase was removed to a new microcentrifuge tube. DNA was subsequently precipitated with cold (-20°C) isopropanol, the mixture centrifuged at 10,000 x *g* for 30 min, and the supernatant discarded. Finally, the pellet was washed with cold (-20°C) 70% ethanol, pelleted again at 10,000 x *g* for 10 min, air dried under the laminar flow and the DNA extract then stored in 50-100µl TE buffer (10m*M* Tris, bring to pH 8.0 with HCl, 0.1m*M* EDTA).

DNA concentration was determined by using a Hoefer Mini Fluorometer while comparing a calf thymus DNA standard (Amersham Pharmacia) against the unknown DNA extract. After calibration of the instrument following the manufacturers instruction, 2µl of DNA extract was mixed with 2ml working solution prepared fresh for the day and analysed. Results obtained were used to prepare PCR-DNA templates of 1ng/µl concentration, achieved by diluting the DNA extracts with double distilled water.

The reagents that were used for DNA preparation, visualisation and PCR reaction were as follows:

<u>Reagents:</u>

- 10x TNE Buffer, pH 7.4

  To 800ml of Milli-Q water the following was added:

100mM Tris	12.1g
10mM EDTA.Na_2	3.7g
2.0M NaCl	116.8g

  pH of 7.4 was adjusted with concentrated HCl, solution then made up to 1l with Milli-Q water and autoclaved or filtered.

- Hoechst 33258 Dye (10mg/ml STOCK)

  20µl STOCK Hoechst 33258 dye was added to 180µl sterile water

<u>Working solution (50ml):</u>

45ml water
5ml 10x TNE
5µl Hoechst dye
Solution was stored in an aluminium foil wrapped Schott-bottle to protect from light.

During later stages of the PhD thesis research, fungal DNA was isolated from either pure cultures or decayed wood samples using the FastDNA® Kit (Qbiogene, Inc., CA) with the provided Cell Lysis Solution-Y (CLS-Y) for fungi according to the manufacturer. Using either fungal tissue or liquid nitrogen fast frozen and mortar pulverised decayed wood chips (stored in sterile plastic tubes with a screw top), 100-200 mg were added to a Lysing Matrix A tube containing ceramic beads and suspended in 1.0ml CLS-Y solution before homogenising in a FastPrep® Instrument for 20 sec at a speed setting of 5.00 three times. In between each homogenisation run, tubes were stored on ice for 2 min and after the third run, centrifuged at 14,000 x *g* for 15 min to pellet debris. About 650µl of supernatant was transferred to a new 2.0ml microcentrifuge tube and an equal volume of binding matrix added and mixed. After storing the tube for 5min at room temperature, it was centrifuged at 14,000 x *g* for 1 min to pellet binding matrix and followed by discarding the supernatant. 500µl Salt/Ethanol Wash Solution (SEWS-M solution; and provided with the FastDNA® Kit) was added and the pellet gently resuspended with the force of expelling and bringing up the liquid into a pipette tip. Centrifuging the tube at 14,000 x *g* for 1 min, the supernatant was subsequently discarded, the tube again centrifuged at the same speed for 10 sec to enable removal of the rest of residual liquid with a small pipette tip. DNA was then eluted by gently resuspending binding matrix in 100µl of DNA Elution Solution in Ultra pure water (DES solution; and provided with the FastDNA® Kit) and incubation for 5 min at 55°C in a heat block. Final centrifugation at 14,000 x *g* for 1 min and transfer of eluted DNA to a clean microcentrifuge tube delivered about 100µl eluted DNA, storable at -20°C for extended periods or 4°C until use.

2.2.6.2 Polymerase Chain Reaction Amplification

For the amplification of the complete internal transcribed spacer (ITS) region, 15µl to 50µl final volume PCR mixtures were prepared following the protocol of White *et al.* (1990). Mixtures contained: 1/10 each of 10X amplification Buffer (15mM $MgCl_2$, 500 mM KCl, 100 mM Tris HCl (pH 8.3), 0.1% gelatine) and dNTP's (2 mM each of dATP, dGTP, dTTP and dCTP), 0.5 units of *Taq* polymerase and 10μM of the primers **ITS1-F (CTTGGTCATTTAGAGGAAGTAA)**, as taken from Gardes and Bruns (1993) and designated as specific for higher fungi, and **ITS4 (TCCTCCGCTTATTGATATGC)** a universal primer after White *et al.* (1990) as positive control, and water to make up 50μl. The two primers ITS1-F and ITS4 were chosen together as it was expected that they would amplify the complete rDNA-ITS region for ascomycetes and basidiomycetes (Jasalavich *et al.* 1998), a preferable situation anticipating, therefore that most fungal species occurring in leaky buildings should be detected.

The following PCR reaction cycles were performed in an Eppendorf Mastercycler gradient 5331: an initial denaturation at 94°C for 3 min followed by 39 cycles of amplification where denaturation at 94°C for 35 sec, annealing at 55°C for 55 sec and extension at 72°C for 2 min had been programmed, and finishing with a final extension at 72°C for 7 min.

PCR products were examined by electrophoresis in 1% (w/v) agarose gels (Ultrapure^{TM} Agarose, Invitrogen, Spain) in 1 x trisboric acid and EDTA (TBE; 0.89M Tris, 0.89M Boric acid and 0.02M Na_2 EDTA) for 60 min at 110 V (volts) and bathing the gel for 10 min in EtBr (ethidium bromide) staining solution to enable visualisation of the DNA bands under UV light for photography (Quantity One 1-D Analysis software, Version 4.4, BioRad).

In addition to the primer pair ITS1-F and ITS4, a set of primers developed by Martin & Rygiewicz (2005) called NSI1 (forward) and NLB4 (reverse) were used to amplify fungal DNA targets from isolated DNA of pure cultures and environmentally decayed wood samples as NSI1 and NLB4 were found to be more discriminating against plant DNA (Martin & Rygiewicz 2005). Using 1μl fungal DNA extract (extraction after FastDNA® Kit as described in Section 2.2.6.1), PCR was performed in 15μl reaction volumes using 1x TAQ amplification Buffer (10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, pH 8.3), 0.5μM of each primer **NSI1 (GATTGAATGGCTTAGTGAGG)** and **NLB4 (GGATTCTCACCCTCTATGAC)**, 0.2mM dNTP, 10mg/ml bovine serum albumin (BSA) and 0.45 Units Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and water to make up 15µl on an Eppendorf Mastercycler gradient 5331 equipped with a thermal lid. Obtained PCR products were purified using the method from Werle *et al.* (1994) by preparing the following reaction mixture: To 5µl PCR product was added 0.5µl (10u) Exonuclease I and 1µl (1u) Calf Intestine Alkaline Phosphatase *or* Shrimp Alkaline Phosphatase, mixing and incubating at 37°C for 15 min and ending the reaction by heating the mixture at 85°C for 15 min. 1µl of the purified PCR products were again amplified, this time with the primer pair ITS1-F and ITS4 by repeating the two steps above. Successfully amplified and purified PCR products were subsequently run in a sequencing reaction.

2.2.6.3 Fungal Species identification

Species identification by DNA sequencing was conducted either at the Cellwall Biotechnology Centre at Scion, Rotorua, New Zealand (1) or at the University of Waikato DNA Sequencing Facility, Hamilton, New Zealand (2). DNA sequence data was generated at both facilities with the Big Dye terminator kit from ABI (Version 1.1 or 3.1 Big Dye Terminator from Applied Biosystems) and the products were run on an ABI 3130 gene analyser (1) or a MegaBACE DNA Analyses System (2) (Amersham Biosciences).

One tube with at least 5µl purified PCR template solution and separately supplied primer tubes at concentrations of 5pMol/µl using primers ITS1-F for sequencing of the forward rDNA-ITS region and ITS4 were used as the control sequences. Sequences were later viewed and aligned with SEQUENCER^{TM} Version 4.8, Gene Codes Corporation, Michigan, USA.

Species identification was based on the results of BLAST searches (Altschul *et al.* 1990) (BLASTn – nucleotide-nucleotide search; Version 2.2.18; Altschul *et al.* 1997) for homology comparison with known sequences in GenBank (housed at the National Centre for Biotechnology Information (NCBI; (http://www.ncbi.nlm.nih.gov/)). A minimum of 95% sequence overlap to an existing ITS sequence of at least 450 base-pairs (bp) in the GenBank database was required for identification. Matches for samples with 96 – 100% similarity with existing species were considered as identified and named to the species level. Those sequences that showed a similarity to existing species of 95% or less were designated names to the genus, family or order. Unmatched sequences were named Unmatched Fungus, Basidiomycete or Ascomycete with their respective bp length.

Further, to outline the significance of a matching sequence, the Expect (E)- value was recorded with BLASTn searches and only those with an E-value of 0.0 thresholds were considered as identified.

2.3 Results and Discussion

2.3.1 Fruiting bodies

In addition to identifications by sequencing (Section 2.3.2), thirteen fungal fruiting bodies were identified by traditional morphological examination through combinational use of identification keys and are listed in Table 2.2. Some exemplifying photographs showing the identified fungi *Gloeophyllum sepiarium*, *Lentinus squamosus*, *Serpula lacrymans* and *Phellinus contiguus,* growing on various building materials, are shown in Figures 2.1 to 2.4. Further, Figure 2.5 outlines the difficulty to analyse a “dark” fruiting body due to its unnatural appearance, thought to belong to *Gloeophyllm sp*. but the molecular investigation couldn’t reveal a positive identification since no PCR amplification of extracted DNA was achieved.

Figure 2.1 Fungal fruiting body *Gloeophyllum sepiarium* growing between two vertical studs (red arrow); detected after exterior wall cladding material was removed.

Figure 2.2 Fungal fruiting body *Lentinus squamosus* detected behind monolithic cladding material. Note: Magenta arrowheads indicating the original positioning of the wall framing (Courtesy of Beagle Consultancy Ltd., Rotorua, NZ).

Figure 2.3 Fungal fruiting body *Serpula lacrymans* development under house floorboards (blue star); Red 'dust' on the ground and on various building materials are reproductive spores produced by this fungus (yellow arrow) (Courtesy of AA House Checks Ltd., Christchurch, NZ).

Figure 2.4 *Phellinus contiguus* detected growing on softwood weatherboards made of *Dacrydium cupressinum* Lamb. (rimu) (Courtesy of Ian Dickie, Landcare Research, Southbridge, NZ).

Figure 2.5 “Dark” fungal fruiting body samples (red arrows) without any prominent identification features; already detached from the framing timber but still connected with a thick and tough fungal mycelium mat (yellow arrow) (Courtesy of Beagle Consultancy Ltd., Rotorua, NZ).

Decay fungi were traditionally identified using keys that differentiated fungi on the basis of fruiting body morphology (Breitenbach & Kränzlin 1986; Ryvarden & Gilbertson 1993), mycelial strand (cord) morphology (Huckfeldt & Schmidt 2006b), or by determination of mycelial morphology and enzyme tests etc. using pure cultures (Stalpers 1978). Precise molecular methods were not available for the identification of indoor wood decay fungi until the 1980s (Schmidt 2007) and many cultures from buildings were misnamed as demonstrated by Schmidt & Moreth (2003). For example, some indoor wood decay fungi such as species of *Antrodia* or *Coniophora* cannot be distinguished at species level in culture (Schmidt 2000). It is for these reasons, as well as speed, that molecular methods are currently being used for more routinely identification purposes, as presented in the following Section 2.3.2.

2.3.2 ribosomal DNA and species confirmation

The fungal diversity occurring in framing timber of New Zealand houses was investigated. The two extraction methods used in this PhD thesis research, the CTAB extraction method after Vogler & Bruns (1998) and the commercial FastDNA® Kit (Qbiogene), for isolating DNA from pure fungal cultures from decayed framing timber delivered enough clean DNA for amplification by PCR. Additionally, the FastDNA® Kit successfully extracted DNA from decayed wood after grinding surface sterilised samples to a powder. Primer pairs ITS1-F (higher fungi specific) and ITS4 (universal primer) achieved good results by amplification of a total of seventy-four fungal DNA extracts, representing the fungal Divisions of sixty-four Basidiomycetes, five Ascomycetes, one Zygomycete and four unknown fungal Divisions (Table 2.1 and 2.2). In twenty-one cases, amplification with these primers was achieved by a nested PCR reaction (Figure 1.20 in Section 1.7.2.3) while first using the primers NSI1 (forward) + NLB4 (reverse) (Martin & Rygiewicz 2005), as exemplified in the ethidium bromide fluorescence of the DNA bands under UV light, separated by electrophoresis gel as photographed using a BioRad camera and shown in Figures 2.6 and 2.7.

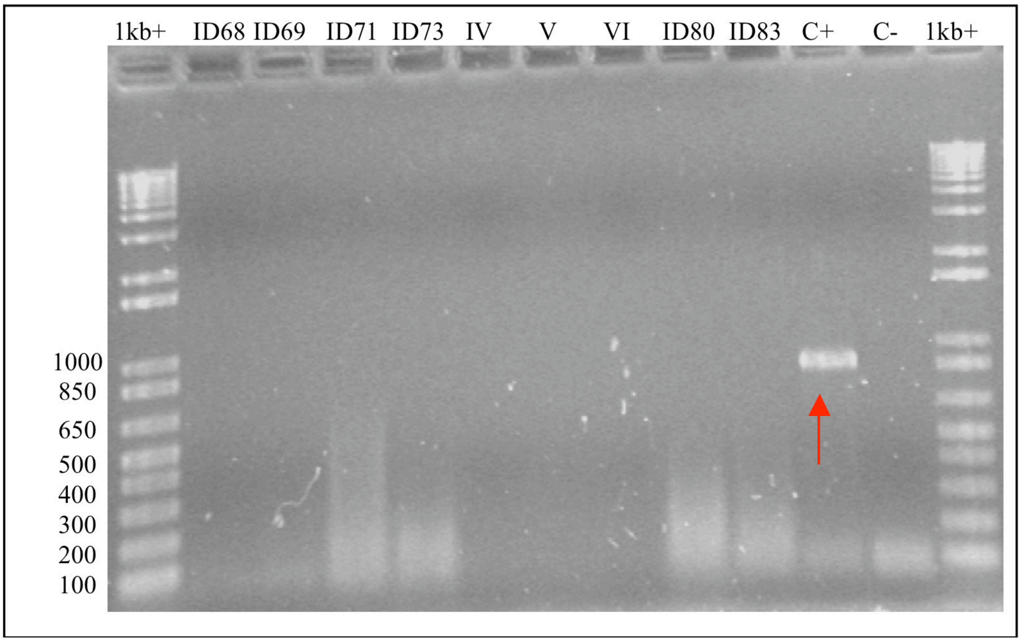


Figure 2.6 Electrophoresis gel result after the first of two nested PCR runs, here with primer pair NSI1 & NLB4, showing the amplification of the positive control (C+ in Lane 11; red arrow), but not the test fungi (Lanes 2 to 10).
For sizing (in bp to the left) the double-stranded DNA product the Ladder 1Kb+ (SolGent Co., Ltd.) was used (Lanes 1 & 13). C- was the negative control (Lane 12).

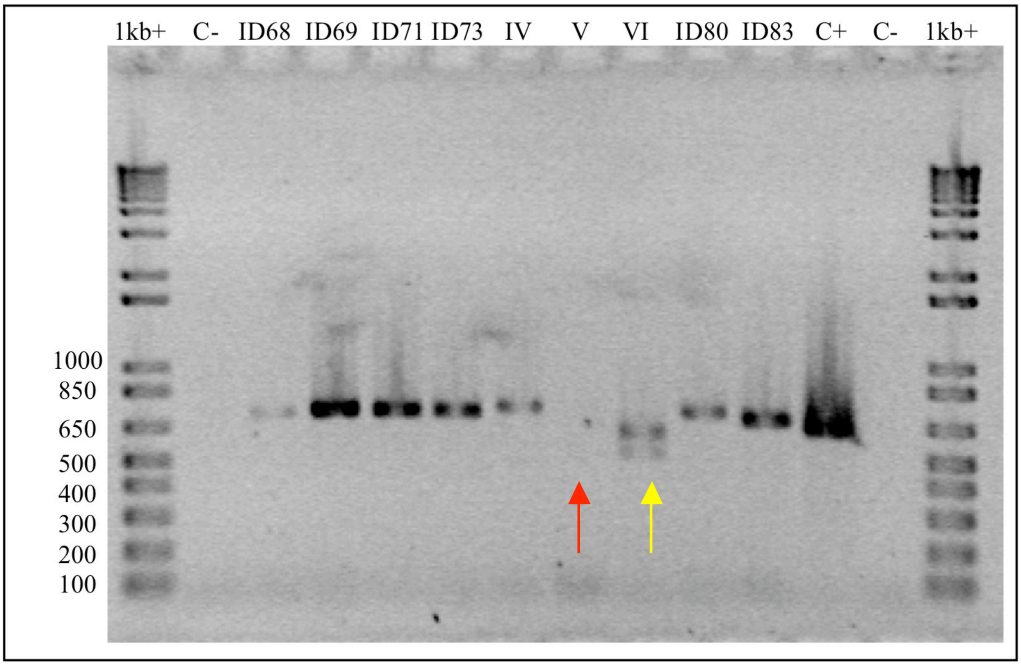


Figure 2.7 Electrophoresis gel result after the second PCR run, here with primer pair ITS1-F & ITS4, now showing the amplification of the test fungi using the original PCR product from result Figure 2.6 (Lanes 3 to 7 and 9 to 11) and the positive control (C+ in Lane 12).
Sample V in Lane 8 (red arrow) was not amplified and sample VI in Lane 9 (yellow arrow) showed two amplifications. The original negative control (C- in Lane 2) was used with one new C- (Lane 13). For sizing (in bp to the left) the double-stranded DNA products the Ladder 1Kb+ was used (Lanes 1 & 14).

A total of 421 samples from leaky buildings, predominantly decayed framing and also fibre cement boards and building paper, were processed. Fifty-five fungal identifications were achieved by molecular techniques using sequence homology comparison by BLAST searching of the GenBank database, additional to the thirteen identifications using classical mycological techniques (Section 2.3.1) and are presented together in Tables 2.1 and 2.2. Sequences identified from these isolates listed had 95% or greater homology with their closest matching sequence. Additionally, four sequences showed a homology to the match of 92 and 93%, nine sequences showed a homology to the match ranging from 79 to 85% and for six sequences no match was found in the GenBank database.

Table 2.1 Results for GenBank queries of fungal species isolated from leaky buildings.

Sample lable	Query sequence length (bp)	GenBank matching sequence Accession number #	Fungus	GenBank score	E-value	Maximum identity (%)	Source
ID3	648	AY618675	*Homobasidiomycete* sp.	1012	0.0	97	culture
ID7	717	AY523813	*Polyporus* sp.	1196	0.0	99	culture
ID9	643	AJ420946	*Gloeophyllum sepiarium*	1086	0.0	99	culture
ID10	697	ASI416068	*Antrodia sinuosa*	1144	0.0	98	culture
ID11	665	AY219367	*Phlebia* sp.	810	0.0	92	culture
ID13	647	AJ420946	*Gloeophyllum sepiarium*	1077	0.0	98	culture
ID16	527	–	unmatched Basidiomycete	–	–	–	culture
ID23	662	AY089732	*Gloeophyllum sepiarium*	1070	0.0	99	culture
ID24	661	AY089732	*Gloeophyllum sepiarium*	1040	0.0	98	culture
ID25	706	AJ249267	*Oligoporus placenta*	1191	0.0	99	culture
ID27	815	AY523813	*Polyporus* sp.	1252	0.0	98	culture
ID28	617	AY497555	*Gloeophyllum sepiarium*	1041	0.0	99	culture
ID29	736	AM902018	uncultured Basidiomycete	1283	0.0	99	culture
ID32	682	DQ912697	*Phanerochaete* sp.	1003	0.0	95	culture
ID33	726	AM902018	uncultured Basidiomycete	1269	0.0	99	culture
ID33B	751	AY251309	*Inonotus* sp.	1029	0.0	93	culture
ID34	709	AJ249267	*Oligoporus placenta*	1196	0.0	99	culture
ID35	708	OPL416069	*Oligoporus placenta*	1182	0.0	99	culture
ID35B	681	AF087484	*Phlebiopsis gigantea*	1124	0.0	98	culture
ID36	702	OPL416069	*Oligoporus placenta*	1184	0.0	99	culture
ID37	701	OPL416069	*Oligoporus placenta*	1177	0.0	99	culture
ID38	598	OPL416069	*Oligoporus placenta*	1054	0.0	98	culture
ID39	597	EU280098	*Trichoderma citrinoviride*	1068	0.0	100	wood
ID40	530	EU402590	*Pycnoporellus* sp.[a]	583	2e-163	85	wood
ID42	573	–	unmatched Fungus	–	–	–	wood
ID43	689	EF126342	*Mortierellales* sp.	1189	0.0	99	wood
ID44	551	DQ404352	*Cadophora* sp.	875	0.0	95	wood
ID56	611	DQ093722	Fungal sp. (Ascomycete)	899	0.0	98	culture
ID57	630	AY497555	*Gloeophyllum sepiarium*	980	0.0	98	culture
ID58	530	EU402590	*Pycnoporellus* sp.[a]	583	2e-163	85	culture
ID59	546	AJ249267	*Oligoporus placenta*	728	0.0	100	culture
ID60	589	AJ420949	*Gloeophyllum trabeum*	1050	0.0	99	culture
ID61	530	EU402590	*Pycnoporellus* sp.[a]	583	2e-163	85	culture
ID63	641	–	unmatched Basidiomycete	–	–	–	culture
ID65	624	AY497555	*Gloeophyllum sepiarium*	1056	0.0	99	culture
ID66	640	AF139961	*Trametes versicolor*	1029	0.0	96	culture
ID67	676	AJ249267	*Oligoporus placenta*	1117	0.0	99	culture
ID68	710	EU402593	*Pycnoporellus* sp.[b]	527	2e-146	79	culture
ID69	682	AJ249267	*Oligoporus placenta*	1162	0.0	98	culture
ID70	530	EU402590	*Pycnoporellus* sp.[a]	583	2e-163	85	culture
ID71	671	AJ416068	*Antrodia sinuosa*	1020	0.0	96	culture
ID72	799	EU689821	uncultured soil Fungus	814	0.0	99	wood
ID73	678	AJ249267	*Oligoporus placenta*	1103	0.0	99	culture
ID74	618	DQ000992	*Pestalotiopsis neglecta*	1040	0.0	98	culture
ID75	549	AY618675	*Homobasidiomycete* sp.	957	0.0	98	culture
ID76	663	AJ416068	*Antrodia sinuosa*	1126	0.0	98	culture
ID77	607	DQ826545	*Resinicium* sp.	863	0.0	93	culture
ID78	667	AJ416068	*Antrodia sinuosa*	1128	0.0	98	culture
ID79	710	EU402593	*Pycnoporellus* sp.[b]	527	2e-146	79	culture
ID80	681	AJ249267	*Oligoporus placenta*	1104	0.0	99	culture
ID81	663	AJ416068	*Antrodia sinuosa*	1113	0.0	98	culture
ID82	710	EU402593	*Pycnoporellus* sp.[b]	527	2e-146	79	culture
ID83	645	AJ420949	*Gloeophyllum trabeum*	1054	0.0	99	culture
ID84	670	DQ826545	*Resinicium* sp.	951	0.0	95	culture
ID86	697	–	unmatched Fungus	–	–	–	culture

(Table 2.1 continued on next page)

Table 2.1 Results for GenBank queries of fungal species isolated from leaky buildings (continued).

Sample lable	Query sequence length (bp)	GenBank matching sequence Accession number #	Fungus	GenBank score	E-value	Maximum identity (%)	Source
ID87	710	AY497555	*Gloeophyllum sepiarium*	1023	0.0	98	culture
ID88	657	AJ416068	*Antrodia sinuosa*	1108	0.0	98	culture
ID89	687	–	unmatched Fungus	–	–	–	culture
ID90	710	EU402593	*Pycnoporellus* sp.[b]	527	2e-146	79	culture
ID91	662	AJ416068	*Antrodia sinuosa*	1113	0.0	98	culture
IV	611	DQ093722	Fungal sp. (Ascomycete)	899	0.0	98	wood
NZFS2207	759	EU734744	*Armillaria hinnulea*	1342	0.0	100	Control culture
Results aerial spore test at a leaky multi-unit building site in Auckland (Chapter 4):							
ASTi	640	AJ416068	*Antrodia sinuosa*	1115	0.0	98	wood
AST2	715	AY461832	*Coprinellus micaceus*	1099	0.0	96	culture
AST3	647	AF255183	*Ganoderma* sp.	1027	0.0	97	culture
AST4	648	DQ912694	*Ceriporia lacerata*	1061	0.0	99	culture
AST5	651	DQ826542	*Resinicium friabile*	971	0.0	97	culture
AST6	675	DQ899094	*Sistotrema brinkmannii*	1132	0.0	99	culture
AST7	661	AF255183	*Ganoderma* sp.	1063	0.0	99	culture
AST8	729	–	unmatched Basidiomycete	–	–	–	culture
AST9	743	AF255183	*Ganoderma* sp.	1012	0.0	97	culture
AST10	653	AF516524	*Polyporus arcularius*	1092	0.0	97	culture
AST11	681	AY966452	*Antrodia* sp.	699	0.0	85	culture
AST12	763	AB084621	*Phlebia uda*	994	0.0	98	culture
AST13	649	AY089736	*Ceriporiopsis* sp.	845	0.0	92	culture

FB – Fruiting body, **bp** – base pairs; **E-value** – Expect value, **a** – Consensus sequence of ID40, ID58, ID61 & ID70; **b** – Consensus sequence of ID68, ID79, ID82 & ID90.

Table 2.2 Fungal identity and associated building materials.

Sample lable	Fungus	Decay type		Associated building material	Location	Previously reported	
						in NZ^{*1}	from buildings^{*2}
ID3	*Homobasidiomycete* sp.	B	–	Bottom of stud	Auckland	–	–
ID7	*Polyporus* sp.	B	BR	Bottom plate	Auckland	✓	✓
ID9	*Gloeophyllum sepiarium*	B	BR	'dark' fruiting body on framing	Auckland	✓	✓
ID10	Antrodia sinuosa	B	BR	Framing	Auckland	X	✓
ID11	*Phlebia* sp.	B	WR	Stud of window sill	Auckland	✓	✓
ID13	*Gloeophyllum sepiarium*	B	BR	Corner stud	Auckland	✓	✓
ID16	unmatched Basidiomycete	B	–	Framing	Auckland	–	–
ID23	*Gloeophyllum sepiarium*	B	BR	Framing	Whangamata	✓	✓
ID24	*Gloeophyllum sepiarium*	B	BR	'dark' fruiting body on boxed pergola beam	Auckland	✓	✓
ID25	*Oligoporus placenta*	B	BR	Joist	Wellington	X	✓
ID27	*Polyporus* sp.	B	BR	Framing	Tauranga	✓	✓
ID28	*Gloeophyllum sepiarium*	B	BR	Corner stud	Tauranga	✓	✓
ID29	uncultured Basidiomycete	B	–	Framing	Auckland	–	–
ID32	*Phanerochaete* sp.	B	WR	Framing	Wellington	✓	✓
ID33	uncultured Basidiomycete	B	–	Garage stud	Auckland	–	–
ID33B	*Inonotus* sp.	B	WR	Garage stud	Auckland	✓	X
ID34	*Oligoporus placenta*	B	BR	Floor joist	Auckland	X	✓
ID35	*Oligoporus placenta*	B	BR	Framing at window corner	Auckland	X	✓
ID35B	*Phlebiopsis gigantea*	B	WR	Stud near bottom plate	Auckland	✓	X
ID36	*Oligoporus placenta*	B	BR	Framing 4th floor	Auckland	X	✓
ID37	*Oligoporus placenta*	B	BR	Framing	Auckland	X	✓
ID38	*Oligoporus placenta*	B	BR	Bottom plate	Auckland	X	✓
ID39	*Trichoderma citrinoviride*	A	Mould	Framing	Auckland	✓	✓
ID40	*Pycnoporellus* sp.	B	BR	ND	Auckland	X	X
ID42	unmatched Fungus	–	–	ND	ND	–	–
ID43	*Mortierellales* sp.	Z	–	ND	ND	✓	X
ID44	*Cadophora* sp.	A	SR	ND	ND	✓	X
ID56	Fungal sp.	A	–	ND	ND	–	–
ID57	*Gloeophyllum sepiarium*	B	BR	ND	ND	✓	✓
ID58	*Pycnoporellus* sp.	B	BR	ND	ND	X	X
ID59	*Oligoporus placenta*	B	BR	ND	ND	X	✓
ID60	*Gloeophyllum trabeum*	B	BR	ND	ND	✓	✓
ID61	*Pycnoporellus* sp.	B	BR	ND	ND	X	X
ID63	unmatched Basidiomycete	B	–	ND	ND	–	–
ID65	*Gloeophyllum sepiarium*	B	BR	ND	ND	✓	✓

(Table 2.2 continued on next page)

Table 2.2 Fungal identity and associated building materials (continued).

Sample lable	Fungus	Decay type		Associated building material	Location	Previously reported in NZ[*1]	Previously reported from buildings[*2]
ID66	*Trametes versicolor*	B	WR	ND	ND	✓	✓
ID67	*Oligoporus placenta*	B	BR	ND	ND	X	✓
ID68	*Pycnoporellus* sp.	B	BR	ND	ND	X	X
ID69	*Oligoporus placenta*	B	BR	ND	ND	X	✓
ID70	*Pycnoporellus* sp.	B	BR	ND	ND	X	X
ID71	*Antrodia sinuosa*	B	BR	ND	ND	X	✓
ID72	uncultured soil Fungus	–	–	ND	ND	–	–
ID73	*Oligoporus placenta*	B	BR	Parapet	Auckland	X	✓
ID74	*Pestalotiopsis neglecta*	A	–	ND	ND	X	X
ID75	*Homobasidiomycete* sp.	B	–	ND	ND	–	–
ID76	*Antrodia sinuosa*	B	BR	Chimney stud	ND	X	✓
ID77	*Resinicium* sp.	B	WR	ND	ND	✓	✓
ID78	*Antrodia sinuosa*	B	BR	ND	ND	X	✓
ID79	*Pycnoporellus* sp.	B	BR	ND	ND	X	X
ID80	*Oligoporus placenta*	B	BR	ND	ND	X	✓
ID81	*Antrodia sinuosa*	B	BR	ND	ND	X	✓
ID82	*Pycnoporellus* sp.	B	BR	Framing	Auckland	X	X
ID83	*Gloeophyllum trabeum*	B	BR	Building paper	ND	✓	✓
ID84	*Resinicium* sp.	B	WR	ND	ND	✓	✓
ID86	unmatched Fungus	–	–	ND	ND	–	–
ID87	*Gloeophyllum sepiarium*	B	BR	ND	ND	✓	✓
ID88	*Antrodia sinuosa*	B	BR	ND	ND	X	✓
ID89	unmatched Fungus	–	–	Mycelium on framing	Auckland	–	–
ID90	*Pycnoporellus* sp.	B	BR	Decking timber	Auckland	X	X
ID91	*Antrodia sinuosa*	B	BR			X	✓
IV	Fungal sp.	A	–	Framing	Auckland	–	–
FB1	*Coprinus domesticus*	B	WR	Bathroom	Auckland	✓	✓
FB2	*Antrodia xantha*	B	BR	Wardrobe	Auckland	✓	✓
FB3	*Gloeophyllum trabeum*	B	BR	On house wall	Auckland	✓	✓
FB4	*Gloeophyllum sepiarium*	B	BR	On monolithic cladding	Auckland	✓	✓
FB5	*Gloeophyllum sepiarium*	B	BR	On monolithic cladding	Hamilton	✓	✓
FB6	*Phellinus contiguus*	B	WR	Weatherboard	Southbridge	✓	✓
FB7	*Lentinus squamosus*	B	BR	Fibre cement	Auckland	✓	✓
FB8	*Trametis versicolor*	B	WR	Weatherboard	Auckland	✓	✓
FB9	*Gloeophyllum trabeum*	B	BR	On fascia board/joint	Auckland	✓	✓

(Table 2.2 continued on next page)

Table 2.2 Fungal identity and associated building materials (continued).

Sample lable	**Fungus**	**Decay type**		**Associated building material**	**Location**	**Previously reported in NZ**[*1]	**Previously reported from buildings**[*2]
FB10	*Gloeophyllum sepiarium*	B	BR	On nog in lounge	Auckland	✓	✓
FB11	*Gloeophyllum sepiarium*	B	BR	ND	ND	✓	✓
FB12	*Gloeophyllum sepiarium*	B	BR	Framing	Auckland	✓	✓
FB13	*Serpula lacrymans*	B	BR	Under floor/ on framing/ onto carpet	Christchurch	✓	✓

A – Ascomycete; **B** – Basidiomycete; **BR** – brown rot; **SR** – soft rot; **WR** – white rot; **Z** – Zygomycete; **ND** – not disclosed location; ***1** – after Landcare Research, Manaaki Whenua, NZFUNGI Database (http://nzfungi.landcareresearch.co.nz); ***2** – after Huckfeldt & Schmidt (2006a) and Ridout (2000).

The four species that were frequently isolated and considered to be common in New Zealand are presented in Table 2.3.

Table 2.3 Common brown rot decay fungi from New Zealand leaky buildings.

Decay fungus	**Frequency**	**Location**
Gloeophyllum sepiarium	13x	Ground floor to roof on framing, pergolas
Oligoporus placenta	11x	Bottom plate to roof on framing
Antrodia sinuosa	8x	Ground floor to roof on framing
Gloeophyllum trabeum	4x	Ground floor to roof on framing, through cracks in monolithic cladding

Prior to this study, little was known about the fungi that caused decay in New Zealand leaky buildings, particularly their identification. Use of polymerase chain reaction (PCR), and subsequent DNA sequencing, as well as classical mycological techniques based on morphology, has provided new identifications and new insights into the relative importance of different decay fungi. With respect to basidiomycete decay fungi, comprising mainly brown rot fungi, it is likely that the data presented in Tables 2.1, 2.2 and 2.3 provides a reliable measure of the most important decay fungi involved, with brown rot fungi being dominant in the tested samples (isolated from 65% of samples), followed by 14%

of samples containing white rot fungi and only 1% of samples containing soft rot fungi.

The two fungal species that were most commonly associated with leaky buildings in New Zealand in this PhD thesis research were the two brown rot fungi *Gloeophyllum sepiarium* (3%) and *Oligoporus placenta* (2%). This result on its own was surprising, since first, generally there was not a dominant fungus found in greater percentage of samples tested, demonstrating a high biodiversity of decay fungi in the samples, and second, *O. placenta* hasn't been recorded previously in New Zealand. However, when observing the presence and percentage present of the two decay fungi from a global perspective, they were associated to be the biggest threat to buildings in the leaky condo crisis during 1990s in Canada (Morris & McFarling 2007) and Huckfeldt & Schmidt (2006a) reported a 1% occurrence for these two decay fungi in buildings in northern Europe (temperate (semi humid) climate zone, and comparable to New Zealand's climate). Hence, it can be suggested that *G. sepiarium* and *O. placenta* play an important role in the degradation of wooden building materials worldwide, and this is confirmed also for New Zealand.

The main decay fungi occurring in buildings in northern and middle Europe are, as of 2006, *Serpula lacrymans* with 31.6% occurrence, *Coniophora* sp. with 26.1% and the white *Polypores* with 11.2% (review of Huckfeldt & Schmidt 2006a). The percentages were based on 17,670 study cases around Europe between 1952 and 2006 and covered 65 different fungal decay species, comprising 65 to 80% brown rot, 26% white rot and 8.4% soft rot fungi. In comparison, this PhD thesis research, even though being on a smaller scale, found the same tendency for brown rot fungi to be dominant at 65%, followed by white rot fungi at 14% and soft rot fungi at 1% (Table 2.2) for 87 isolates comprising 33 different decay fungi from leaky building materials. Further, 15 of the 33 decay fungi listed in Table 2.2 were previously reported as occurring in buildings (Huckfeldt & Schmidt 2006a; Ridout 2000). This study differed from Europe, though, that the highest percentage found repeatedly of a single fungal species was 3%, a point that will be further discussed in Chapter 6.

Concerning the unidentified fungi in Table 2.1, there was one basidiomycete that was frequently isolated from the wood sample range, macroscopically indicating a brown rot typical decay pattern. Comparing the obtained sequences within BLAST on GenBank of the DNA from this isolate, it delivered a sequence homology ranging from 79 to 85%, which is not high enough to give an identification, but with the closest match to the fungal genus of *Pycnoporellus sp*. Fungi belonging to this genus are generally referred to as brown rot polypores (Family: polyporaceae) and concerning molecular investigations are grouped phylogenetically within the *Antrodia* clade of polypores (Lindner & Banik 2008). *Pycnoporellus fulgens* was the original match according to a BLAST search (85% confidence), however, this fungus is not reported from New Zealand but is closely related to the Australasian fungus *Rigidoporus laetus* (Hood 1992) of which no sequence was available in the GenBank database. Since the fungus was isolated eight times from independent wood samples in this PhD thesis research, it is noteworthy and it can be suggested that the list of the four common brown rot decay fungi in New Zealand leaky buildings (Table 2.3) will most likely be extended in the future with a fifth common, but yet unknown brown rot fungus, probably originating from the Australasia region.

The finding that the four most important basidiomycete decay fungi were brown rot fungi is not surprising bearing in mind that New Zealand uses soft wood framing. Brown rot decay fungi are also the predominant cause of decay in the Northern Hemisphere where softwood is used as the main source of interior structural timber (Schmidt 2007). The most important physiological factors affecting decay in buildings are wood moisture content and temperature (Sections 1.5.1 and 1.5.3). Zabel & Morrell (1992) described how the organisational levels of the cell wall affect the relationships between water and wood. The gross capillary zone of wood consists of a lumenae of cells and pit cavities, which can contain water as a liquid and/or vapour. The transient capillary zone consists of the non-crystalline cellulose zones of microfibrills, which contain bound water not available to decay fungi. The critical point for wood fungi is the fibre saturation point, which is approximately 30% moisture content (as a percentage of the oven dry weight of wood).

Huckfeldt & Schmidt (2006a) reported the minimum moisture content for decay by *G. sepiarium* and *G. trabeum* as 30% and 31%, respectively, with range for growth between 46 and 207% as shown in laboratory decay test results using *Pinus sylvestris,* where the minimum moisture content needed for the fungi to actively decay the timber to achieve a weight loss of 2-10%. *Antrodia* species need moisture above the fibre saturation point, which only occurs after direct exposure of the timber to water (Schultze-Dewitz 1985 in Schmidt 2007). In occupied and heated buildings, Grosser (1985) reported equilibrium wood moisture contents (EMC) of 6 to 15% and 12 to 21% for weather protected outside constructions in Europe, which are unsuitable for decay fungi. However, equilibrium moisture contents in the range of 16-20% can be a warning of potential problems and for EMC between 20-25%, serious problems are likely.

In a laboratory test by Dirol & Vergnaud (1992), *Pinus sylvestris* (Scots pine) blocks were exposed to prolonged condensation and they recorded moisture contents as high as 45% after 50 hours. Furthermore, they suggested that non-ventilated framing timbers in buildings might reach similar moisture contents after 50 hours; for example, when water build-up following condensation after consecutive winter nights fails to dry out due to low daytime temperatures.

Another study by Viitanen & Ritschkoff (1991) divided decay damage in Finnish houses into three categories: 54.5% of decay occurrence was attributed to water leakages, 28.8% to construction defects and 16.7% to insufficient ventilation of subfloor- and roof spaces. The suitable wood moisture content range for growth of brown rot fungi was reported as 30-70%.

In terms of temperature, wood decay fungi isolated from buildings have been found to grow well under laboratory conditions between 20 and 37.5°C (Huckfeldt & Schmidt 2006a). This broad temperature range includes the common temperatures that occur in buildings. Additionally, growth has been recorded for ‘house decay’ fungi in the laboratory from 0 to 44°C (Eslyn 1986; Humphrey 1933; Schmidt 1994). Of the four most important brown rot decay fungi in this study, *G. sepiarium* and *G. trabeum* have reported growth optima within the range 28-38°C, whereas *A. sinuosa* and *O. placenta* have slightly lower temperature optima within the range 25-31°C and 25-28°C, respectively (Huckfeldt & Schmidt 2006a). However, this study did not test the reflection of temperature to location within a building and type of fungi identified. Huckfeldt & Schmidt (2006a) found *Gloeophyllum* spp. occurred mainly in wood associated with windows, doors and roofs in Europe. In this PhD thesis research, similar occurrences were found on framing (ground floor to roof) associated with pergolas and framing behind faulty monolithic cladding.

Oligoporus placenta was found to occur on flooring material, staircases and windows (Huckfeldt & Schmidt 2006a), whereas in this study it was found in a bottom plate and in roof framing. For *Antrodia sinuosa*, Huckfeldt & Schmidt (2006a) noted occurrences in cellar and roofing framing. Findings from this study included framing from the ground floor to the roof, including the isolation of *A. sinuosa* under a roof junction. Earlier studies by Schultze-Dewitz (1990; in Schmidt 2007) where *Antrodia* species were found in the attic and upper floor of houses in eastern Germany, concur with the findings of this investigation. Schmidt (2007) emphasised the importance of recording the location within a building where decay fungi were isolated.

3 Laboratory decay tests of wood preservative-treated *Pinus radiata* D. Don

3.1 Introduction

Four fungal isolates found to be in the greatest occurrence in New Zealand leaky buildings (as presented in Chapter 2) were tested for their capability to decay treated and untreated timber. Standard laboratory wood block decay tests were performed to determine their susceptibilities and tolerances towards common wood preservatives used in timber constructions. Capability to decay was determined by mass loss (ML) of test blocks following a standard laboratory decay test. Two decay tests were conducted for both replication and to evaluate in the second trial additional fungi

3.2 Material and Methods

3.2.1 Wood block decay tests and fungal isolates tested

The method used was the standard "Sutter jar" method to test the efficacy of wood preservatives and has been successfully used at Scion, Rotorua, NZ since the 1980s. This mycological testing originates from Sutter (1978), who advocated the use of plastic Petri dish containers instead of glass Kolle flasks and recommended thin (1-4 mm) cross-section samples, since they were found to lead to the same results as samples cut parallel to the surface (Sutter 1978). The test uses similar principles to those used in the European standards EN-113 and BS 6009:1982 but differs in exposure time to the decay agent.

3.2.1.1 "Sutter jar" test protocol

Preservative treated (Section 3.2.2) and untreated controls of *Pinus radiata* D. Don and *Fagus silvatica* (Beech) sapwood blocks (35 (tangential) x 35 (radial) x 7 (transverse) mm) were used. Additional *P. radiata* controls included those treated with water and organic solvent only. All samples were cut on a circular saw from air-dried timber, labelled, conditioned to equilibrium moisture content (EMC) of

12 % (T= 20-22°C and RH= 69-72%) and initial weights taken. Samples were then either preservative treated (Section 3.2.3), with some treated wood blocks additionally thereafter being exposed to an accelerated leaching procedure (Section 3.2.3.1), or used untreated. All treated samples, whether leached or not leached were then reconditioned to 12% EMC and re-weighed, before all samples were sent to Schering-Plough Animal Health, Upper Hutt, NZ to be sterilised by gamma irradiation consisting of two runs of 26.0 kGy (kilogray) in sealed sterilisation bags. Petri dish containers were gas sterilised in an air-tight chamber (*AN74* manufactured by H.W. Anderson Products Inc., NC 27258 USA) using *Anprolene Sterilizing Ampoules* (97% ethylene oxide) following the manufacturer's protocol. Each container was then filled with ca. 60ml of autoclaved 2% MA medium under sterile conditions, sealed with a plastic lid containing a centred cotton bud (for aeration), left to settle over night and then inoculated with the test decay fungi isolates (as given in Table 3.1) under a laminar flow. The four test fungi isolates originated from decayed framing timber and were identified using a molecular approach, as presented in Chapter 2, whereas the two control fungi were obtained from the fungal culture collection held by Scion, Rotorua, NZ.

Table 3.1 Pure cultures used to test decaying capability.

Trial ID numbers	Test fungi	Culture-strain source
A, G, M	*Oligoporus placenta*	Isolate ID25
B, J, P	*Coniophora puteana*	Control CP114/Scion
C, F, L	*Antrodia sinuosa*	Isolate ID10
D, H, N	*Gloeophyllum sepiarium*	Isolate ID13
I, O*	*Gloeophyllum trabeum*	Isolate ID60
E, K*	*Serpula lacrymans*	Control NZFS 950/Scion

* These two fungi were used in the second decay test only.

Two inocula of approximately 5 mm² were placed face down on the MA and the container given an identification tag, coding the fungus, preservative treatment, preservative retention and timber species. Afterwards, the jars were stored at 26°C for ca. two weeks until the fungus had covered the agar surface. A sterile 75mm diameter and 1mm thick plastic mat, perforated with 17 evenly distributed 9mm diameter holes, was placed onto the mycelium to prevent direct contact of the wood blocks with the agar. Two test blocks were then placed into each container and exposed to the decay fungi for 12 weeks. There were 10 replicate wood blocks per fungus, treatment and retention. After the exposure period, wood blocks were freed from any adhering mycelium, air-dried for three days, before conditioning to EMC of 12% and final weighing.

Mass loss was calculated based on weights taken at EMC of 12%, which was usually attained after 14 days at T= 20-22°C and RH= 69-72% before and after exposure to decay using the formula:

$$\text{Mass loss (\%)} = \frac{\text{mass before} - \text{mass after}}{\text{mass before}} x100$$

The exact mass loss data to each of the results charts in Section 3.3 is provided in Appendix 2.

3.2.2 Wood preservatives

The preservatives used and shown in Table 3.2 were approved for use in New Zealand (NZS 3640:2003) for Hazard Classes H1 and/or H3 (see Table 3.2 and Section 1.8.2 for definition of hazard classes) and were made available by Koppers Arch Wood Protection (NZ) Limited as concentrates, free of additives such as waxes and resins that are often incorporated into commercial products. However, the boron concentrate supplied contained an isothiazolinone (anti-mould) additive. With the exception of two preservatives, copper azole (CuAz) and IPBC, retentions chosen were the required minimum retention for the particular hazard class, one-third and one-ninth of this retention. With CuAz, a base retention of 0.3% m/m was selected rather than the H3.2 specified 0.23% m/m because it was considered more suitable based on decay test results using copper as the active ingredient (Kamden *et al.* 1995 and 1996; Green & Clausen 2005). With IPBC, a base retention of 0.10 % m/m was selected rather than the H1.2 specified 0.025 % m/m because the literature suggested that the toxic limits for IPBC against most decay fungi are between 0.05-0.16 % m/m (Hansen 1984; Xiao & Kreber 1999). In addition, two concentrations 1/3 and 1/9 of the base retentions were chosen as not being inhibitory to fungal growth. Treatment specifications and concentration calculation are given in Appendix 2.

Table 3.2 Wood preservatives and retentions.

ID number	Wood Preservative	Retentions in (% m/m)[*3]	Active Ingredient	NZ Hazard Class required retentions
BO	Boron[a)]	0.40; 0.13; 0.04	Boric Acid	H1.2[*1] 0.40 % m/m
CuAz	Copper Azole[a)]	0.30; 0.10; 0.03	Metallic Copper and Tebuconazole	H3.1[*2] 0.2288 % m/m
Azole	Tebuconazole & Propiconazole (1:1)[b)]	0.06; 0.02; 0.007	Tebuconazole & Propiconazole	H3.1 0.06 % m/m
IPBC	3-Iodo-2-Propynyl Butyl Carbamate[b)]	0.10; 0.03; 0.01	IPBC	H1.2 0.025 % m/m

[a)] Water as carrier system.
[b)] Solvent as carrier system (Fuelite, Eagle Chemicals, Rotorua, NZ).
[*1] H1.2 applies to timber used in situations protected from the weather, but where the risk of moisture content conductive to decay (Section 1.8.2).
[*2] H3.1 applies to timber used in situations above ground, exposed to the weather – generally in non- structural applications, but including cladding used as bracing if the component is painted. Beams and cladding used as bracing are examples of structural applications of H3.1 (Section 1.8.2).
[*3] (% m/m) retention= % mass per unit mass; i.e.: 0.4% m/m BAE = 2.2kg/m³ at wood density of 550kg/m³ (BAE= Boric Acid Equivalent).

3.2.3 Treatment process

Treatments were carried out in ascending order from the lowest to the highest retentions to minimise waste product using vacuum/pressure treatment plant adapted from an American Sterilizer (manufactured under licence by Atherton's Pty Ltd., Melbourne). Two protocols were established (Table 3.3), one for the water carrier system and one for the solvent carrier. The main difference between the water-system protocol and the solvent-system protocol was that with the latter, the solution was introduced into the treating vessel after vacuum, and that no pressure was applied. Uptake was calculated by weighing the wood blocks before and immediately after treatment.

Table 3.3 Treatment protocols.

Carrier system	**Mean Uptake of treatment solution per Block**	**Vacuum**	**Pressure**	**Fixation**
Water	5.45 g	15 min @ -80kPa	20 min @ 200kPa	2 weeks
Solvent	3.60 g	20 min @ -80kPa	30 min soaking	n/a

3.2.3.1 Leaching procedure

Blocks treated to the base (highest) retention were exposed to an accelerated ageing (leaching) procedure in accordance with BS5761 Part2:1980/EN84 for two weeks before being subjected to the decay trial. In particular, wood blocks were submerged in beakers of distilled water contained in a vacuum desiccator fitted with a stopcock and pressure gauge capable of maintaining a pressure of 4 kPa for 20 min. After this cycle, the wood blocks were left to stand in the beaker for 2 h before replacing with fresh distilled water at a ratio of approximately 5 volumes of water to 1 volume of wood. For the test blocks used in this study, ca. 50ml per block was needed. Distilled water was then exchanged for 14 days at 1-day intervals.

After treatment and fixation or accelerated ageing procedure, three days of airing and 14 days conditioning at 12 % EMC (T= 20-22°C and RH= 69-72%) blocks were weighed before sealing them in sterilisation bags for gamma irradiation as described in Section 3.2.1.1 above.

3.2.4 Differences between Decay tests I and II

A second decay test was conducted in the following year after the Decay Test I results were analysed. There were two differences to the second decay trial, designated Decay Test II:

- Six test fungi were evaluated instead of four by adding one new NZ isolate from the PhD thesis research and a second control fungus.

- The second trial was carried out during winter months, whereas the first trial was carried out during summer months. Given that both tests were fully conducted in the laboratory with the same wood blocks, the season of the year was not considered to be a significant variable.

The wood blocks used in both trials were all prepared and preservative treated at the same time prior to the first test. The blocks needed for Decay Test II were stored at EMC room conditions until required.

3.3 Results and Discussion

3.3.1 Mass loss for control tests wood blocks

Mass losses (ML) for water control blocks, organic solvent control blocks and pine and beech control blocks are presented in Table 3.4.The water control blocks inoculated with *A. sinuosa* at ~ 62% for both Decay Tests I and II gave the maximum mass loss observed for any of the fungi. The next two most significant ML samples were the test fungus *C. puteana*, at about 40% ML, with good agreement between the two trials and *O. placenta* with average 43.5%, with not as good agreement of the data between the two trials, which ranged from 39.8 – 48.9%. It was considered that the test conditions were near the optimum for *A. sinuosa* and *C. puteana*. *O. placenta* caused on average 13% less ML during the second trial, whereas *G. sepiarium* caused substantially, on average 19%, higher ML during the second trial. Possible reasons for this discrepancy are discussed in Section 3.3.4. *S. lacrymans* showed the least ML of any of the tested fungi, at 17.5% on the water control blocks. ML results for all fungi tested with organic solvent and water treated blocks compared to untreated *P. radiata* blocks, showed that the treatment carrier systems on their own were not inhibitory to decay.

Table 3.4 Mass loss results for control samples.

Fungus		**Water Control (ML in %)**	**Solvent Control (ML in %)**	**Pine Control (ML in %)**	**Beech Control (ML in %)**
O. placenta	I	48.9 ± 5.6	49.3 ± 5.9	47.2 ± 5.0	51.9 ± 4.4
	II	39.8 ± 12.0	30.8 ± 5.5	35.3 ± 7.8	37.9 ± 5.4
C. puteana	I	40.6 ± 4.4	38.5 ± 3.6	34.9 ± 2.9	42.2 ± 3.5
	II	39.9 ± 6.4	35.3 ± 6.9	35.0 ± 5.9	40.8 ± 5.0
A. sinuosa	I	61.9 ± 3.7	58.0 ± 3.3	58.8 ± 2.9	50.7 ± 6.3
	II	62.0 ± 5.5	59.6 ± 4.2	54.7 ± 9.5	51.8 ± 5.4
G. sepiarium	I	13.7 ± 3.1	14.9 ± 4.3	16.1 ± 4.2	14.0 ± 1.8
	II	34.4 ± 6.2	34.2 ± 5.4	31.2 ± 5.4	32.9 ± 4.0
G. trabeum	II	18.9 ± 3.0	13.8 ± 3.9	11.2 ± 1.9	24.7 ± 3.6
S. lacrymans	II	17.5 ± 4.8	17.3 ± 4.4	14.6 ± 4.2	15.4 ± 3.1

I – Results after first decay test; **II** – Results after second decay test.

3.3.2 Mass loss for preservative-treated samples

The results of the wood block Decay Tests are presented in graphical form in Figures 3.1 to 3.8, and illustrate the decay capability of the four fungi *Oligoporus placenta* (**OP**), *Gloeophyllum sepiarium* (**GS**), *Antrodia sinuosa* (**AS**), *Gloeophyllum trabeum* (**GT**) isolated during this PhD thesis research and the two control fungi *Coniophora puteana* (**CP**) and *Serpula lacrymans* (**SL**). The specific results for each preservative after the two decay tests are summarised as follows:

CuAz (Figures 3.1 and 3.2)

- *O. placenta, C. puteana*, *A. sinuosa* and *S. lacrymans* had approximate toxic threshold values (defined as the maximum preservative retention for which mass loss of blocks is ≤ 3% mass loss) between 0.3 and 0.1% m/m. Respective ML at 0.1% m/m for these four fungi (26, 8, 10 and 5%) was less than half that which occurred at 0.03% m/m (54, 38, 58 and 19%) which showed that there was a well defined dosage response and indicated that the actual toxic threshold was closer to 0.1 than 0.3% m/m. This was confirmed with the second decay test, where *C. puteana* and *A. sinuosa* caused low ML of 6.0 and 2.1, respectively, at 0.1% m/m CuAz. Further, mass loss results for *O. placenta*, *C. puteana* and *A. sinuosa* were generally in agreement between the two trials.

- The toxic threshold for *G. sepiarium* and *G. trabeum* was substantially less than occurred for the other four fungi, at less than 0.03% m/m (lowest retention tested) indicating that these two fungi of the same genus were particularly susceptible to CuAz. Alternatively the other 4 fungi may have exhibited tolerance to this preservative.

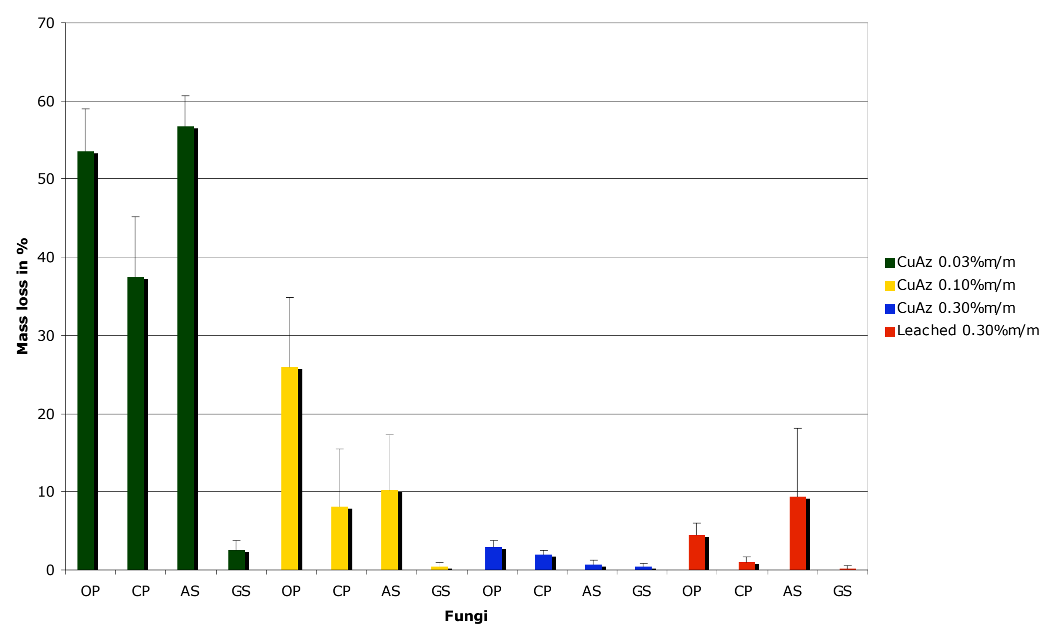


Figure 3.1 Mass loss results for copper azole (CuAz) in Decay Test I.

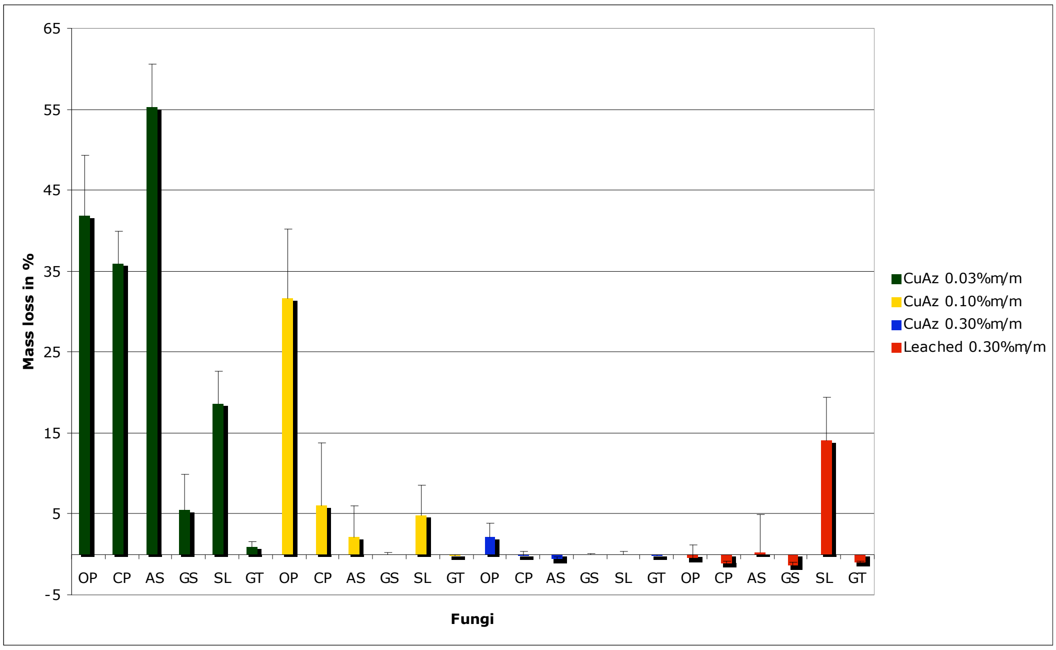


Figure 3.2 Mass loss results for copper azole (CuAz) in Decay Test II.

Boron (Figures 3.3 and 3.4)

- *O. placenta* had an approximate toxic threshold value higher than the highest chosen retention of 0.4% m/m (ML ≥ 5.2%) after the first decay test. After the second decay test *O. placenta* was still the only fungus exhibiting some tolerance at retention of 0.4% m/m, however to a lesser degree (ML of 2.2%).
- *C. puteana*, *A. sinuosa, G. trabeum* and *G. sepiarium* had approximate toxic threshold values between 0.4 and 0.13% m/m. ML at 0.13% m/m was less than a third (3, 5, 1 and 2.8%) that which occurred at 0.04% m/m (30, 30, 16 and 35%) which showed that there was a well defined dosage response and indicated that the actual toxic threshold was closer to 0.13 than 0.4% m/m. However, for *G. sepiarium* this was only revealed after the second decay test where ML of 35% occurred at the lowest retention tested (as opposed to ML of 6.8% after the first decay test).
- *S. lacrymans* had a toxic threshold closer to retention 0.04% (ML of 7.2%) than 0.13% m/m (ML of 0.2%) and this was substantially less than occurred for the other five fungi.

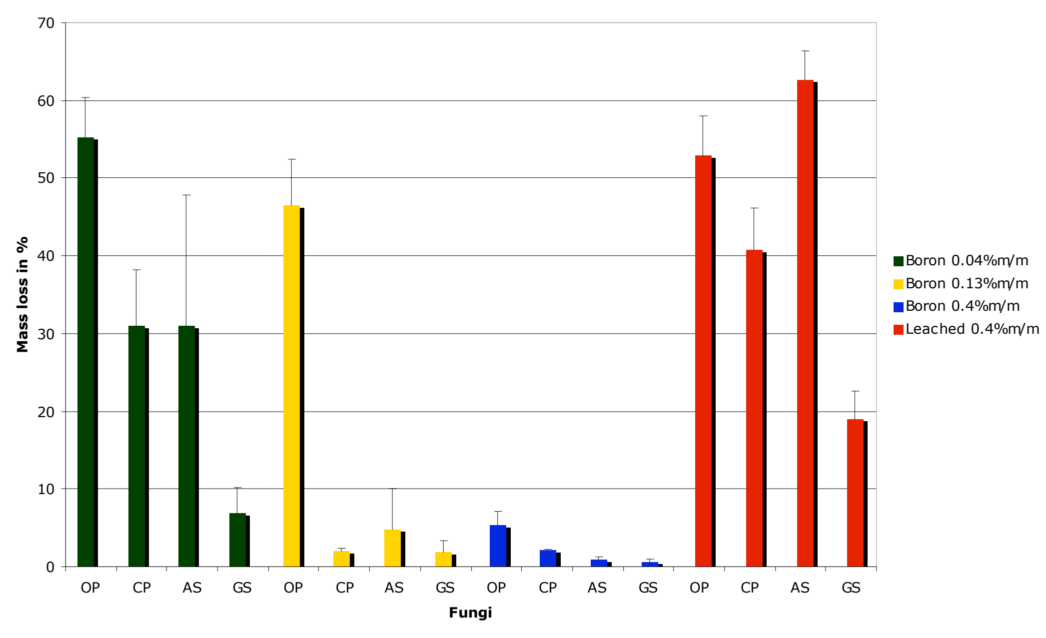


Figure 3.3 Mass loss results for boron in Decay Test I.

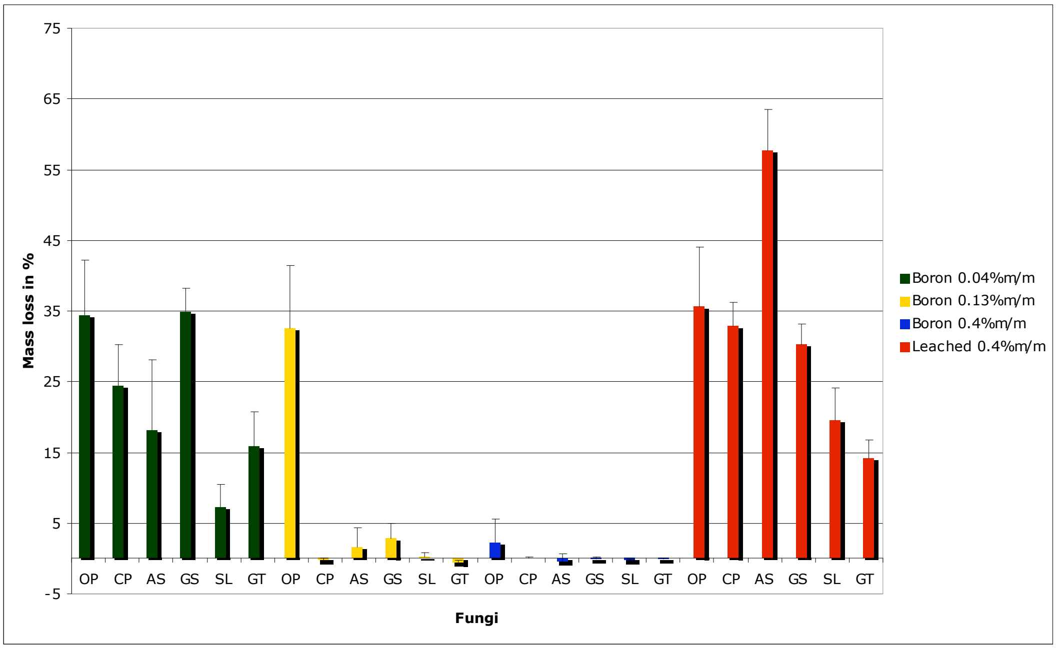


Figure 3.4 Mass loss results for boron in Decay Test II.

IPBC (Figures 3.5 and 3.6)

- *C. puteana* and *A. sinuosa* at retention 0.03% m/m caused highest mass losses of all test fungi ranging between the two decay tests of 23 to 25% and 11 to 19%, respectively.
- The toxic threshold for the control fungus *C. puteana* was fractionally above the highest chosen retention of 0.1% m/m after both decay tests (ML of 3.1 and 6.2%).
- *O. placenta* and *A. sinuosa* had approximate toxic threshold values between 0.1 and 0.03% m/m. ML at 0.03% m/m was less than half (6.7 and 18.7%) that which occurred at 0.01% m/m (35.4 and 47.1%) which showed that there was a well defined dosage response and indicated that the actual toxic threshold was closer to 0.03% m/m than 0.1% m/m.
- *G. sepiarium, G. trabeum* and *S. lacrymans* were more susceptible to IPBC than the other 3 fungi with a toxic threshold between 0.03 and 0.01% m/m, indicated by ML of 18, 13 and 9% respectively at 0.01% m/m. Especially *G. sepiarium* showed a greater tolerance to the lowest retention tested (0.01% m/m) after the second decay test (ML of 18% compared to 4% after first test).

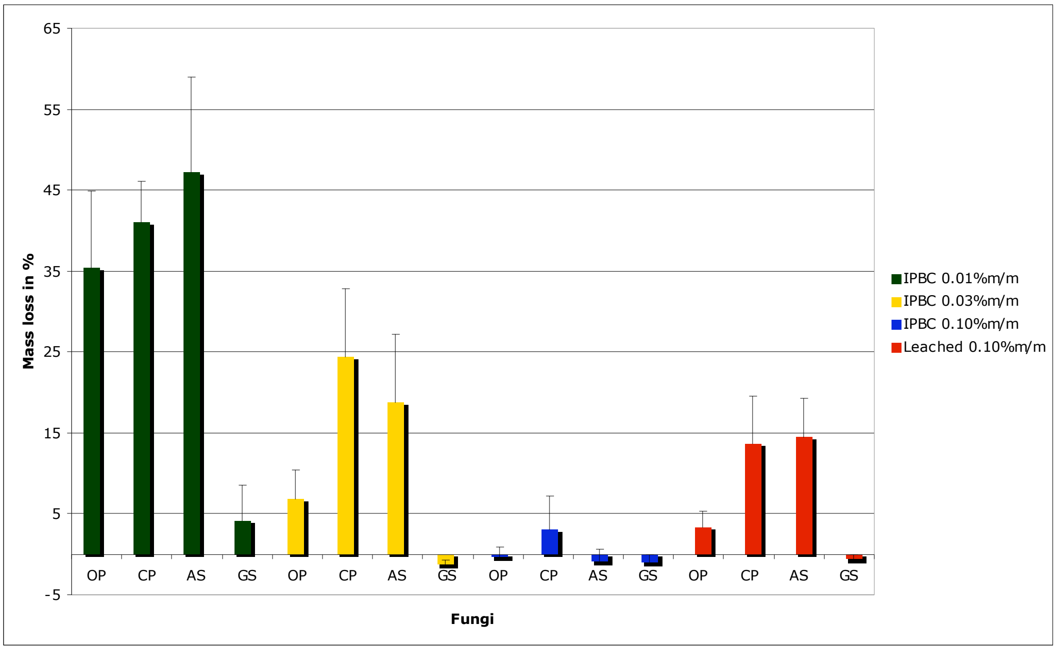


Figure 3.5 Mass loss results for IPBC in Decay Test I.

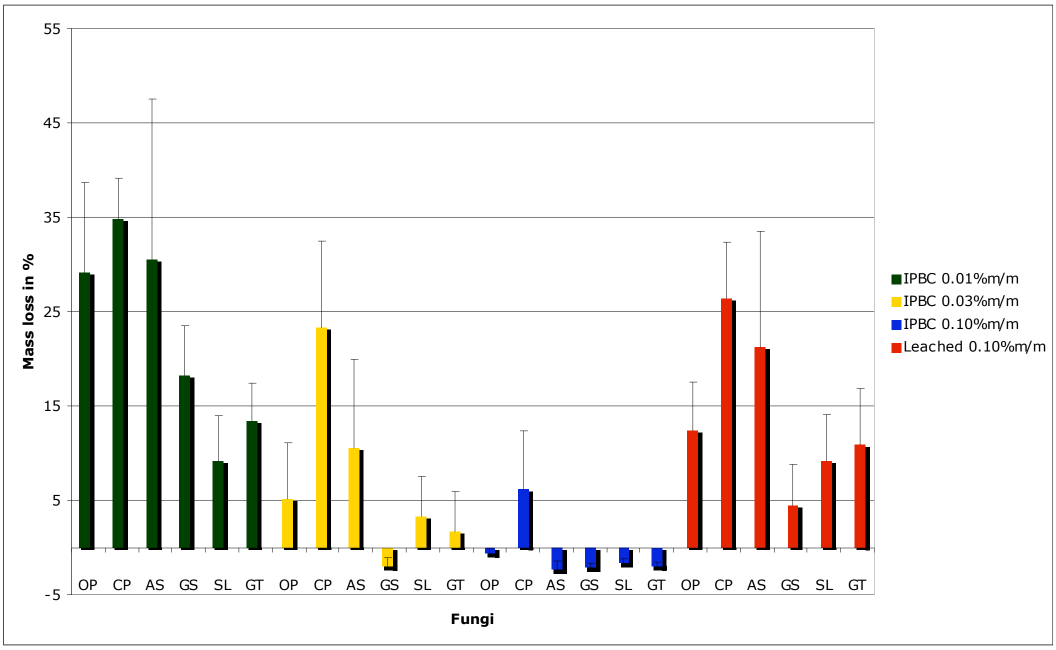


Figure 3.6 Mass loss results for IPBC in Decay Test II.

***Tebuconazole plus Propiconazole* (1:1)** (Figures 3.7 and 3.8)

- *S. lacrymans* had an approximate toxic threshold value higher than the highest chosen retention of 0.06% m/m (ML ≥ 5.4%). Surprisingly, ML caused at the lowest retention tested of 0.007% m/m increased only to ≥ 13.1% ML. Nevertheless, even overall ML caused was low, the control fungus *S. lacrymans* was the only one exhibiting tolerance to the azoles treatment at the highest retention of 0.06% m/m.
- The toxic thresholds for *O. placenta, A. sinuosa* and *C. puteana* were between 0.02 and 0.06% m/m. However, inconsistent ML results between the two decay tests for these three fungi might indicate a toxic threshold closer to 0.06% m/m.
- For *G. sepiarium and G. trabeum* the toxic threshold was less than 0.007% m/m.

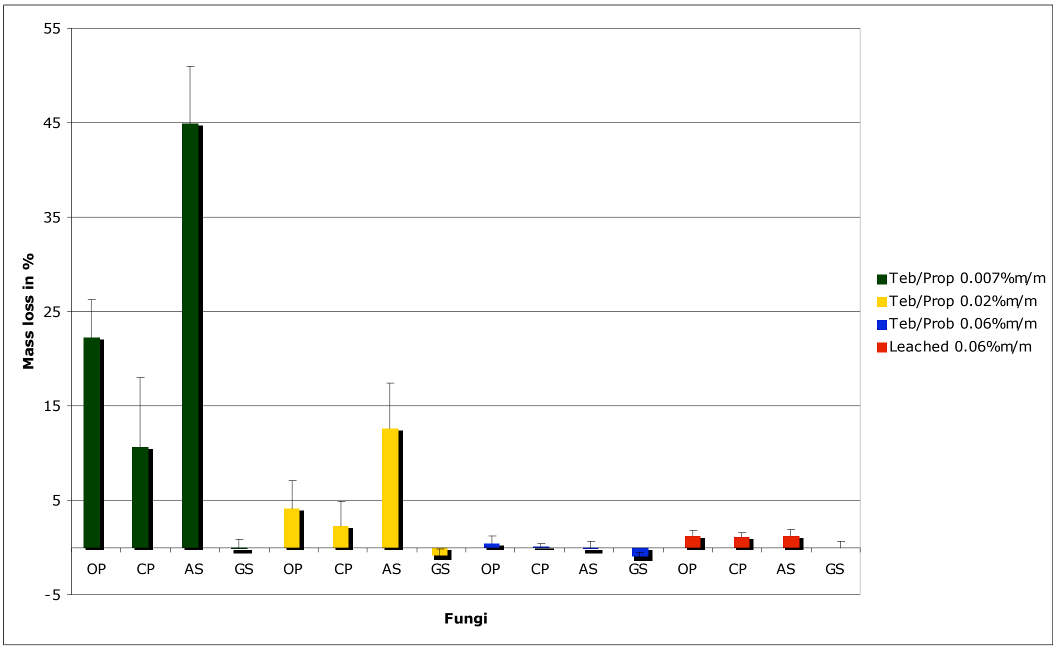


Figure 3.7 Mass loss results for tebuconazole plus propiconazole (1:1) in Decay Test I.

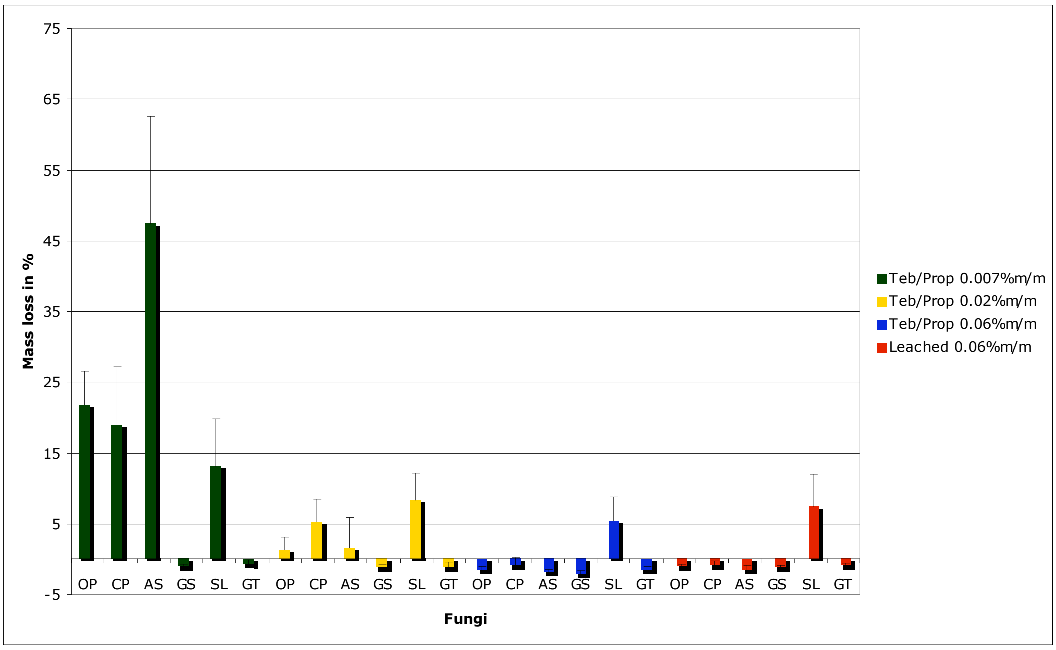


Figure 3.8 Mass loss results for tebuconazole plus propiconazole (1:1) in Decay Test II.

3.3.3 Effects of leaching

- Boron (0.4% m/m) was highly susceptible to leaching with mass losses between 14 and 62.6% across all 6 test fungi, which based on mass losses for the lowest known retention tested (0.04% m/m) indicated complete loss.
- IPBC (0.1% m/m) was substantially less susceptible to leaching than boron but significant losses from leaching was indicated with mass losses from decay ranging between 4.5 to 26%. These results further indicated that the residual retention was closer to 0.03 than 0.1% m/m, i.e. in the order of 50% loss, although this should be considered somewhat speculative in the absence of IPBC analysis.
- Leaching of CuAz (0.3% m/m) caused elevation of ML for *O. placenta* (4.4%) and substantial elevation of ML for *A. sinuosa* (9.3%) and *S. lacrymans* (14.1%).
- Mass loss data for tebuconazole plus propiconazole indicated that these actives are not susceptible to leaching which tends to suggest that it was the copper only that was lost from CuAz. This is further supported through the following two observations:

a. *S. lacrymans* caused similar ML in tebuconazole plus propiconazole treated blocks at retentions 0.02 and 0.06% m/m (ML of 5.4 and 8.3%) and in the leached 0.06% m/m blocks (ML of 7.4%).
b. *S. lacrymans* caused no ML at the highest CuAz retention of 0.30% m/m, but caused 18.6% ML in blocks with the same retention leached for two weeks.

3.3.4 General discussion

The two laboratory wood decay tests showed that the four decay fungi, *Oligoporus placenta*, *Antrodia sinuosa*, *Gloeophyllum sepiarium* and *Gloeophyllum trabeum,* isolated from NZ leaky buildings, were capable of degrading untreated and preservative treated *Pinus radiata* wood blocks. In addition to their frequent occurrences (Chapter 2), the mass loss results of the two decay trials showed their ability to cause considerable damage (mass loss of up to 62% in control blocks for *A. sinuosa*) within a short time frame of 12 weeks under near optimum growth conditions. This clearly demonstrates the potential threat these fungi can pose to framing in water-compromised buildings.

In particular, it was found that *O. placenta* was the most preservative-tolerant fungus which corroborates earlier findings of, for example, tolerance towards copper and zinc compounds (AWPA E10-91). *O. placenta* caused up to 53% ML at the lowest CuAz retention of 0.03% m/m. It was the only fungus causing some ML close to the toxic threshold of ≤3% mass loss at the highest retention of 0.30% m/m, indicating the effectiveness of the organic co-biocide tebuconazole in CuAz against *O. placenta*. An earlier study using a number of arsenic-free copper formulations, including a copper azole (azole not named) and testing these with copper tolerant brown rot fungi such as *O. placenta* and *A. sinuosa*, resulted in minor ML of ≥ 5% at approximate retention of 0.4% CuAz m/m in Southern Yellow Pine (SYP) (Green & Clausen 2005), which supports the observations made after the two decay trials in this PhD thesis research.

For the test fungus *O. placenta* and boron treated *P. radiata*, Drysdale (1994) and Peylo (2005) showed threshold values against fungal decay of around 0.11% m/m

BAE. However, in the current study, retention of 0.13% m/m BAE was susceptible to *O. placenta* with a mass loss of up to 46.4%. Boron at 0.4% m/m gave close to complete control of the test fungi but when leached gave zero protection with ML close to that of untreated *P. radiata*. The boron treatment further inhibited decay of the wood blocks by *S. lacrymans*, which is consistent with the importance of boron treatments to control *S. lacrymans*, in temperate European, buildings, not only as a timber preservative application, but also as a treatment injected into masonry to stop its spread (Schmidt 2006). Somewhat surprisingly, the strain of *S. lacrymans* used showed some copper sensitivity, whereas this fungus was earlier regarded as being a copper tolerant brown rot fungus (Hastrup *et al.* 2005).

The tebuconazole plus propiconazole treatment was found to be the most effective in this test, and was essentially resistant to leaching under the test conditions employed. Only *A. sinuosa* caused high mass loss in wood blocks containing this treatment with 47.1% ML at retention 0.007% m/m, although the genus *Antrodia* is usually known as being copper tolerant (Duncan 1958; Green & Clausen 2005). The current study recorded up to 55% mass loss for *A. sinuosa* in CuAz at a retention of 0.03% m/m. According to Buschhaus & Valcke (1995) the most difficult fungus to control with tebuconazole plus propiconazole was the brown rot *O. placenta* with toxic thresholds between 0.024 and 0.039% m/m for solvent based formulations and for all basidiomycetes they suggest a toxic threshold of 0.056% m/m. This corroborates results of this study where the toxic threshold for *O. placenta* were between 0.02 and 0.06% m/m and this dosage was similarly effective against all the test fungi except the control fungus *S. lacrymans*. This particular fungal strain exhibited a tolerance towards this treatment with a toxic threshold greater than the highest tested retention of 0.06% m/m. Buschhaus & Valcke (1995) who used most of the fungi tested here with this treatment, did not test *S. lacrymans* and so far no other toxic threshold data for this fungus/treatment combination could be found in the literature. Nevertheless, comparing mass loss caused by *S. lacrymans* at the lowest retention of 0.007% m/m (ML 13.1%), this was close to the average ML caused in control samples with 14.6%, indicated some tolerance under the test conditions employed. According to reports by Schultz & Nicholas (2003) and Archer & Lebow (2006), the tebuconazole plus propiconazole combination is effective against most decay fungi, but has limited efficacy in controlling sapstain, mould fungi and insects/termites. Hence the azoles are often used in combination with other biocides, such as copper azole treatment (Schultz & Nicholas 2003).

The control fungus *C. puteana* caused up to 7% ML in IPBC treated pine at 0.1% m/m, and up to 34% ML at retention of 0.03% m/m which is close to the NZS3640:2003 H1.2 retention of 0.025% m/m. Hedley (2000) reported that *C. puteana* caused a mass loss of 9.8% at retention 0.024% m/m IPBC + 0.006% m/m permethrin. Further, Hedley (2000) showed in the same study that permethrin at this concentration alone does not inhibit this fungus. Permethrin is an insecticide normally added to IPBC formulations for commercial above-ground end-uses, since IPBC alone is ineffective against insects (Archer & Lebow 2006). Muin & Tsunoda (2003) tested IPBC in wood composites, such as MDF or plywood and found a toxic threshold of 0.37% m/m to the white rot fungus *T. versicolor* (isolated two times from leaky buildings during this PhD thesis research, Chapter 2) and *Fomitopsis palustris*. While further studies are required, this result suggested that the NZS3640:2003 H1.2 minimum IPBC retention should be substantially increased.

G. sepiarium and *G. trabeum* were the most susceptible isolates to all four wood preservatives, particularly to CuAz and tebuconazole plus propiconazole, with *G. trabeum* known to be copper-sensitive (Humar *et al.* 2006). *G. sepiarium* and *G. trabeum* only caused significant mass losses in blocks treated to each of the respective lowest retention for treatments IPBC 0.01% m/m (ML up to 18 and 13% respectively) and Boron 0.04% m/m (ML up to 35 and 16% respectively). In untreated control samples, *G. sepiarium* initially caused less mass loss than the other test fungi (≥ 16%) after the first decay trial and very similar to the ML achieved by *G. trabeum* (≥ 19%) after the second trial. However, after the second decay trial the same *G. sepiarium* fungal strain caused up to 34% ML in control samples, similar to ML results by *O. placenta* (≥ 47%) and *C. puteana* (≥ 35%). It was initially suspected that the low mass losses for *G. sepiarium* and *G. trabeum* may have been a reflection of the test temperature of 26°C, which is low compared to the optimum growth temperature range reported for these two fungi of 33-35°C (Eslyn 1986 and Table 1.2 Chapter 1, Section 1.5.3).

Furthermore, Huckfeldt & Schmidt (2006a) tested *G. sepiarium* and *G. trabeum* on untreated *Pinus sylvestris* sapwood and found that they required a minimum moisture content of 30% to cause more than 2% mass loss, and more than 46% moisture content to cause more than 10% mass loss in 8-12 weeks. They further listed mass loss data for *G. sepiarium* of 12-21% and for *G. trabeum* of 27-69% after 3 to 5 months at 20°C which indicates that it was not the test temperature but the moisture content of the wood which was not favourable to these decay fungi and that there can be a great variability between tests using the same fungal strain.

This variability between trials was further shown with the test fungus *O. placenta*, causing 13% less ML in control blocks during the second trial, but still with ML of 35% well within the range earlier recorded by Wälchli (1977) of 25 to 49% after 8 to 16 weeks and 28°C using *Pinus sylvestris*.

Negative mass loss results for the following were recorded and were attributed to lack of consistency in EMC room conditions:

- *O. placenta* and *G. sepiarium* in CuAz, IPBC and tebuconazole plus propiconazole;
- *A. sinuosa* and *G. trabeum* in all four treatments;
- *S. lacrymans* (control fungus) in IPBC and boron;
- *C. puteana* (control fungus) in CuAz, tebuconazole plus propiconazole and boron treated blocks.

Whilst these laboratory data do not necessarily reflect in-service performance, they demonstrate substantial differences in efficacy of preservative formulations currently approved for framing treatments in New Zealand and possible deficiencies where framing may be subjected to severe leaching, or where inadequate retentions are used.

4 Aerial spore test at a multi-unit leaky building

4.1 Introduction

In this part of the PhD research study, the objective was to determine if aerial spores could be detected in leaky buildings; if so, were they viable and culturable and whether they could be subsequently identified. The objective was accomplished by sampling at a multi-unit leaky building complex in Auckland, NZ internal air, wall cavity air and exterior air using a Merck MAS-100 instrument, which enabled collection of aerial spores directly onto various selective media.

Indoor and outdoor air sampling is commonly used to determine spore concentrations (viable and non-viable spores) with respect to potential health hazards to both flora and fauna, including humans. In addition, fungal spores in the air are implicated in the damage of food commodities and in the deterioration of organic materials and stored products (Pyrri *et al.* 2007). If not managed correctly, the interior environment potentially provides a range of microclimates suitable for fungal growth if favourable temperatures, nutrients, oxygen and water are present. A previous New Zealand study of fungi on moisture compromised interior wall surfaces reported 77% common airborne fungi, 16% toxigenic fungi and 7% wood decay fungi, (Waipara 2002 and 2003). However, little is known about the effects of moisture compromised wall cavities on internal and local external air spore counts or of the origin of spores from decay fungi that eventually caused decay in buildings.

The study sought to isolate and identify fungi from decayed framing materials at a leaky building site, to compare these with aerial spore trapping data from the same building site and to determine if viable fungal spores could be captured using different selective media plates. It was carried out at an Auckland building site comprised of 51 two-story units which were built from winter to spring of 2001. Eighteen months after construction was completed, defects in the monolithic cladding material were noticed, although capping and flashing faults were identified even earlier. Behind these defects, moisture readings of the framing ranged between 80-90% moisture content (MC). Initial remediation practices were applied but many of the units were left un-repaired. Therefore, these units were regarded as suitable for this aerial spore study because there was a high probability that decayed framing and active fungal growth would be present.

4.2 Materials and Methods

4.2.1 Sampling locations

Sampling of aerial spores was done on two consecutive days in late spring 2007 in Auckland, New Zealand, during an average temperature of 21°C and moderate wind conditions. Three different sampling spots were chosen to test for fungal spores, as given in Table 4.1:

Table 4.1 Aerial spore sample spot locations.

	Sample spot	**Distance from Ground (m)**	**Distance from Building/effected wall (m)**	**Different Locations**
A	Outside	0.5	1.5	4
B	Wall cavity air	1.5-2.5	0	4
C	Inside	0.5	2.0	3

A- Air samples were taken 1.5m away from the building and away from the leak affected wall with parts of the cladding opened up before samples were taken (Figure 4.1).

B- Air samples were taken from wall cavity air just after the cladding had been cut open to gain access to the framing, allowing only enough space for the Merck MAS-100 instrument to sample cavity air (Figure 4.1).

C- Air samples were taken inside three units in the centre of one room, 2m away from the affected leaky wall, downstairs and at the same location on the 1st floor. All windows had been closed for 15 min before sampling of the internal air took place.

Figure 4.1 Left: *Gloeophyllum sepiarium* fruiting body on framing stud and associated white mycelium. Centre: outside sampling ground level. Right, upper: sampling within wall cavity. Right, lower: decayed and damp wall framing.

4.2.2 Air sampling

A total of sixteen air samples were collected at each sampling spot and location, using the air sampler Merck MAS-100 Eco®. This volumetric sampler, shown in Figure 4.1, aspirates air through a perforated plate containing 400-holes positioned over a 90 mm Petri dish containing agar growth medium. The air-stream is at a fixed speed of 100 litre/min; air can be collected for variable periods of time according to the settings on the air sampler. The MAS-100 air sampler compensates for all factors that could influence the airflow, such as volume of agar in the Petri dish or the variation in Petri dish diameter. The sampler was operated at calibrated flow rates and sampling was within air volume ranges of 10 to 200 litre of air. Air samples were cultured on 90mm Petri dish plates containing either Yeast-Malt agar, Malt agar, Carboxymethylcellulose medium or limited medium, which is semi-selective for basidiomycetes. After completion of air sampling, the plates were removed from the sampler, labelled in the order in which they were sampled, sealed with parafilm and stored in a plastic box container with a lid. Upon arrival back at the laboratory, the plates were incubated at room temperature and examined every three days and the final number of colonies formed on the agar plates counted after four weeks. The final number of colonies were adjusted using the positive hole conversion table provided by the manufacturer of the MAS-100; the conversion is based upon the presumption that as the number of viable particles being impinged on a given plate increases, the probability of the next particle going into an empty hole decreases, calculated using the basic formula after Feller (1950): $Pr = N[1/N+ 1/N-1+ 1/N-2.....1/N-r+1]$ (Pr= probable statistical total; N= total number of holes in the sampling head (=400)). Results are expressed as colony forming units per cubic metre (CFU/m^3) with Figure 4.2 exemplifying a typical colony plate.

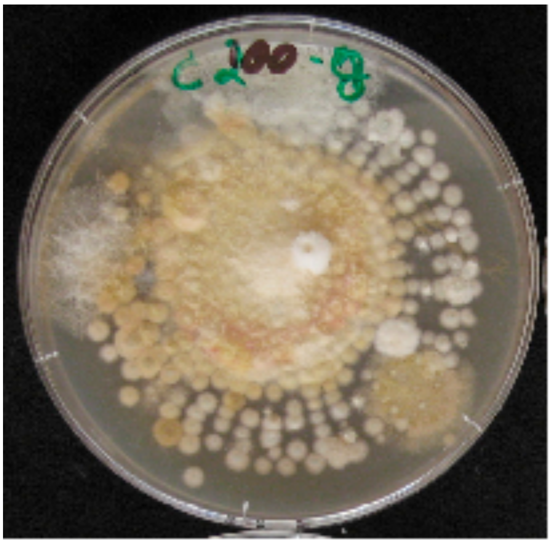


Figure 4.2 Petri dish of cultured fungal colonies after four weeks incubation in the laboratory with sampling 200 litres of wall cavity air.

4.2.3 Media and Reagents

The following four media and reagents were used for the aerial spore study:

Malt Yeast Extract Agar **(YM)** – 1.5% malt extract (DANISCO Ltd., Manukau City, Auckland, New Zealand), 0.2% yeast extract (OXOID Ltd., Basingstoke, Hampshire, England), 1.5% agar (DANISCO Ltd., Manukau City, Auckland, New Zealand) in distilled water.

Basidiomycete semi-selective agar **(BMA)** (Worrall 1991) – 1.5% malt extract, 0.2% yeast extract, 1.8% agar, 0.2 g/l chloramphenicol (Sigma-Aldrich Inc., St. Louis, USA), 0.06 g/l benlate, 0.1 g/l streptomycin sulphate (Sigma-Aldrich Inc., St. Louis, USA), 2 ml/l lactic acid (APS Ajax Finechem, Australia) in distilled water.

Carboxymethylcellulose (*Trichoderma viride*) medium A **(CMC)** – 14 ml 10% $(NH4)_2$ SO_4 (BDH Laboratory Supplies, England), 15ml 1M KH_2PO_4 (BDH Laboratory Supplies, England), 6 ml 35% urea (APS Ajax Finechem, Australia), 3 ml 10% $CaCl_2$ (BDH Laboratory Supplies, England), 3 ml 10% $MgSO_4.7H_2O$, 1 ml Trace elements solution (10 ml concentrated HCl (APS Ajax Finechem, Australia), 0.51% $FeSO_4$ (APS Ajax Finechem, Australia), 0.186% $MnSO_4.4H_2O$ (BDH Laboratory Supplies, England), 0.166% $ZnCl_2$ (J.T. Baker USA), 0.2% $CoCl_2$ (BDH Laboratory Supplies, England)), 2 ml Tween 80 (BDH Laboratory Supplies, England), 0.2% Carboxymethylcellulose (Sigma, USA) and 1.5% agar in distilled water.

Malt Agar **(MA)** – 1.5% malt extract, 2% agar in distilled water.

4.2.4 Screening for cellulase activity of colonies formed on Carboxymethylcellulose medium

Air samples collected onto Carboxymethylcellulose (CMC) (*Trichoderma viride*) medium A and their subsequent colony-forming units were screened for their ability to degrade carboxymethylcellulose. On this medium, the actual numbers of colonies formed were noted after four weeks of incubation at 20°C room temperature and later compared to the total number of CMC positive colonies. Adapting the protocol of Teather & Wood (1982), after 4 weeks incubation at room temperature, plates were incubated at 37°C for 24 hours and then flooded with an aqueous solution of Congo red (1mg/ml) and allowed to react for 30 minutes. The Congo red solution was then poured off and the plates were subsequently flooded with 1M NaCl for 60 minutes. If cellulose activity was present, this was seen as a clear zone around the fungal colony. The diameter of the clear zone and the colony was measured in two dimensions at 90° to each other and the values averaged. An Index of Relative Enzyme Activity (RA) was calculated by dividing the total area of activity (the area of the clearing zone less the area of the colony) by the area of the colony. This RA index provides a gross measure of the production of enzymes for the degradation of a given substrate (Bradner *et al.* 1999).

4.2.5 Wood sampling

Wood samples were chiselled out of framing at units where the cladding had been removed and earlier wall cavity air samples had been taken. The chisels were washed with 90% ethanol prior to each sampling to avoid cross contamination from the different sample locations. Using gloved hands, the wood samples were examined for visible fungal decay and stored in sealable plastic bags. In the laboratory, wood samples were surface sterilised by immersion in 70% ethanol, flamed, and then plated on 2% MA or BMA media at room temperature ranges of 20 to 24°C. Pure cultures were then established by inoculation of hyphal tips.

4.2.6 Fungal identifications

Pure fungal cultures were identified using PCR and DNA sequencing of the amplicons as described in Chapter 2 and are included in Table 2.1, Section 2.3.2. Further, some isolates were tentatively identified using the illustrated descriptions to genera of imperfect fungi after Barnett (1960).

4.3 Results and Discussion

Air sampling at leaky building units detected viable fungal aerial spores at every of the three sampling locations and full data sets of aerial spore counts are provided in Appendix 3.

4.3.1 Exterior Samples

The average CFU values for all three sampling locations are presented in Table 4.2. The series range, which is the difference between the highest and the lowest result of a test series, for all 80 samples taken outside, was 1140 CFU/m^3. YM and MA media recorded the highest airspore counts of 456 and 458 CFU/m^3, respectively, and BMA recorded the lowest of 24 CFU/m^3. Fungi isolated from exterior air samples were mainly *Penicillium* sp. and some unidentified white fungi and yellow or pink yeast-like fungi. Four pure cultures were obtained from this exterior spore sampling location and after PCR and DNA sequencing of the amplicons, the following basidiomycetes were identified:

- *Coprinellus micaceus* (commonly known as Ink cap),
- *Polyporus arcularius* (a decay fungus usually associated with forests),
- *Sistotrema brinkmannii* (a decay fungus previously reported growing on wet, wood-framed windows and walls in Sweden and Norway (Flannigan *et al.* 2001; Alfredsen *et al.* 2005) causing neither brown- nor white- rot (Huckfeldt & Schmidt 2006a)), and
- *Ganoderma* sp. (hardwood and conifer decay fungus).

4.3.2 Wall cavity Samples

The average wall cavity CFU/m^3 count of 3714 for MA medium and 172 for BMA medium were eight and seven times, respectively, the values recorded for the exterior sampling location. Results for all four media are given in Table 2. The series range for all 48 samples was 8555 CFU/m^3. Fungi isolated from these wall cavity air samples were *Penicillium* sp., *Fusarium* sp., *Epicoccum* sp., *Gliocladium* sp., *Streptomyces* sp. and *Paecilomyces* sp. and attached to a sample of wood taken from a stud, a fruiting body sample of *Gloeophyllum sepiarium* was identified (Figure 4.1).

Alongside the fruiting body was a white strand-forming fungus. Dry mycelium was isolated from this fungus, but no successful molecular identification was obtained. A second white mycelium and pore-forming fungus was isolated at a different unit from a stud beneath the roof junction.

Successful culturing enabled molecular identification of this second mycelium, resulting in a sequence of the polypore fungus *Antrodia sinuosa* with a 98% match compared with sequences available using the Basic Local Alignment Search Tool (BLAST) available online at GeneBank. In addition, two pure cultures were obtained from the aerial spore sampling and successful sequencing identified the basidiomycetes *Ganoderma* sp. and *Resinicium friabile* with 99% and 97% homology level, respectively. *R. friabile* is known to cause decay of wood and bark of hardwood and conifers and it is the only known *Resinicium* species to occur in the southern hemisphere (Nakasone 2007)).

4.3.3 Interior Samples

The interior of the building unit samples gave results which showed the highest CFU/m^3 of 581 recorded for MA medium whereas the lowest CFU/m^3 of 42 was recorded for BMA medium (Table 4.2). The series range for all 96 air samples taken in the interior was 1600 CFU/m^3. Fungi isolated from these inside locations were *Penicillium* sp. and *Fusarium* sp. Six pure isolates obtained from the internal aerial spore test identified the following five fungal basidiomycete species after successful sequencing: *Ganoderma* sp., *Ceriporiopsis* sp. (wood decay fungus), *Phlebia uda* (hardwood decay fungus), *Ceriporia lacerata* (white rot decay fungus) and *Antrodia* sp.

Table 4.2: Average colony-forming units per m^3 for air samples taken inside, outside and out of wall cavities of leaky units.

Sample media	Inside (CFU/m^3)	Outside (CFU/m^3)	Wall Cavity (CFU/m^3)
CMC	367	361	587
BMA	42	24	172
YM	529	456	1798
MA	581	458	3714

CMC- Carboxylmethylcellulose (*Trichoderma viride*) medium;
BMA- Basidiomycete semi-specific agar;
YM- Malt Yeast Extract agar;
MA- Malt Agar.

4.3.4 Number of Carboxymethylcellulose degradative fungi

Colonies formed on CMC media plates sampled outside, inside and from wall cavities were screened for carboxymethylcellulase activity using Congo red staining solution (Teather & Wood 1982). Colonies formed on plates sampled inside showed the greatest carboxymethylcellulase activity of 12.9%, compared to 7.7% and 2.9% for exterior and wall cavity locations respectively (Table 4.3 and Appendix 3). When the index of relative enzyme activity (RA) was taken into account, interestingly, the highest activity of 0.65 (RA) was recorded for the colonies formed on the wall cavity plates, followed by 0.52 (RA) for samples taken outside and the lowest of 0.45 (RA) for the inside samples, showing the reverse compliment to the total percentage of CMC positive colonies at each location (Table 4.4 and Appendix 3). This phenomenon might find its reason, in case of the wall cavity air samples, in the overall condition of the water-compromised wall. Examination of the framing revealed wet (ca. 80% MC) timber with signs of decay (Figure 4.1) and indicating the presence of fungi capable of degrading cellulose. Therefore, it was considered a high likelihood that spores would be present that showed a significant index of relative enzyme activity (RA) in wall cavities. However, only two colonies from a total of 143 carboxymethylcellulose positives showed an index of relative activity equal or greater than 1 in this screening study, suggesting that the overall capability for degradation of carboxymethylcellulose was small for the fungal colonies isolated from aerial spores and measured.

Table 4.3: Number and percentage of Carboxymethylcellulose positive fungal colonies.

	Inside	**Outside**	**Wall cavity**
Number of fungal colonies tested for CMC activity	556	701	585
Number of fungal colonies demonstrating clearing of CMC	72	54	17
% of total colonies that were CMC positive	12.9	7.7	2.9

Table 4.4: Index of relative enzyme activity.

	Inside	**Outside**	**Wall cavity**
Average RA ± S.D.	0.45 ± 0.17	0.52 ± 0.18	0.65 ± 0.18

4.3.5 General Discussion

The aerial spore study in Auckland resulted in successful aerial spore testing, as 224 culture plates were screened positive for fungal colonies. More than fifty sub-cultures were derived. DNA was extracted from twelve pure cultures and amplicons from PCR were DNA sequenced. Common aerial fungal spore species included *Penicillium*, *Fusarium*, *Gliocladium*, *Epicoccum* and *Paecilomyces*, all of which have been reported in prior aerobiological studies (Waipara 2003; Pyrri 2007; Kasprzyk *et al.* 2006; Morales *et al.* 2006; Vesper *et al.* 2005). A study by Phipps *et al.* (2005) of air quality in 33 domestic houses in Wellington, NZ found the following fungal species indoors: *Cladosporium* (85%), *Penicillium* (10%), (dominant species indoors and outdoors), *Aspergillus*, *Mucor*, *Botrytis, Alternaria*, *Paecilomyces*, *Gliocladium*, *Phialophora*, *Epicoccum*, *Fusarium*, *Ulocladium* and *Geotrichum*. In this study *Penicillium, Paecilomyces*, *Gliocladium*, *Epicoccum* and *Fusarium* were identified at this multi-unit leaky building site.

This study focussed on the detection of viable fungal spores or hyphal fragments (diaspores) indoors, outdoors and within the leaky wall cavities, their identification and comparison with identifications from framing. Of the twelve pure cultures sequenced from the air-analysis study, eleven were identified as basidiomycetes. Two of these identifications, *Ganoderma* sp. and *Coprinellus micaceus*, are common aerial spore producers. A study into airborne basidiospores in the atmosphere of Seville, Spain identified the species *Coprinus* as the most frequently isolated and *Ganoderma* as frequent (Morales *et al.* 2006). *Ganoderma* was also found in the outdoor air in front of typical single-family homes (non-leaky) in Cincinnati, USA by Lee *et al.* (2006). Kasprzyk *et al.* (2006) found spores in outdoor urban and rural air environments in Poland, and Chakraborty *et al.* (2000) detected *Ganoderma* in indoor air in Burdwan, India. In this PhD thesis research, *Ganoderma* sp. was found in the outdoor air, indoors and within the wall cavities.

The brown rot decay fungus *Antrodia sinuosa* was isolated and identified from framing as described in Chapter 2 and one pure culture was found from an interior air sample, which was amplified, sequenced (681 base pairs) and putatively identified as *Antrodia* sp. though the overall match for the genus *Antrodia* was only 85%. It would be highly interesting to probe for some other genes in this isolate and determine its phylogenetic relationship to *A. sinuosa.*

The determined CFU/m^3 values were approximately the same for interior (581) and exterior air (458). The interior and exterior series range values were 1600 and 1140, respectively. None of the fungi isolated were regarded as toxigenic, although the interior spore concentrations might be considered a potential health risk. Burge (1996) suggested that if indoor levels are two times the outdoor level, or greater than 1,000 CFU/m^3, then a source for mould amplification should be suspected. The mean CFU/m^3 value on a rich media (MA) in wall cavities (3714) was eight times the exterior value of 458 and 6.5 times the value of 581 for the interior air. Phipps *et al.* (2005) recorded a similar mean for indoor and outdoor air of 350 CFU/m^3 values for 33 houses in New Zealand although the selection criteria for the houses was not related to leakage issues.

Whilst use of the basidiomycete semi-selective agar (BMA) enabled isolation of *Antrodia* sp., in general, use of the four different media did not substantially improve separation of fungal types and use of media other than MA did not increase the gross number of CFU/m^3 recorded. Nevertheless, use of CMC media provided valuable insight into the frequency of occurrence of fungi capable of degrading CMC and their importance relative to the total number of CFU/m^3 recorded. Use of CMC media recorded 143 CMC positive colonies, with the highest number of 72 isolated from inside air samples, followed by 54 colonies found from outside samples and only 17 colonies found inside the wall cavities. This result showed that at the time of sampling, viable fungal spores capable of CMC degradation were present at every sampling location but it is difficult to account for the differences between locations. Interestingly, the index of relative enzyme activity (RA) was found to be below 1 for all positive colonies except for two, which indicated that the capability of the colonies formed to actually degrade carboxymethylcellulose was low. An explanation for the different amount of CMC-degrading isolates between the three samples may be that the conditions (sampling locations) were not equivalent in having fungi capable of producing aerial spores.

In summary, viable fungal spores including mould fungi, basidiomycetes and yeasts were captured from the air using an air sampler and selective media. Further, two of the identified common and important brown rot decay fungi, namely *G. sepiarium* and *A. sinuosa*, as described in Chapter 2, were isolated from this multi-unit leaky building site. A *G. sepiarium* fruiting body, isolated from framing timber, was molecularly identified, as was *Antrodia* sp., the only decay fungus isolated from both framing and air samples. Overall, the combinational approach of wood sampling and aerial spore sampling provided useful information about the species variation at this multi-unit building complex.

5 Micromorphological changes in *Pinus radiata* D. Don with brown rot fungal colonisation

5.1 Introduction

Decay fungi can pose a threat to *Pinus radiata* timber products such as kiln dried framing since they have evolved the means to decompose large volumes of wood completely (Schwarze 2007). Brown rot decay fungi in particular are known to rapidly cause structural failure, making early detection crucial. Techniques often applied for this detection involve, for example, destructive mechanical strength tests such as measurement of modulus of elasticity (MOE) and rupture (MOR), electrical conductivity (i.e. moisture meter), acoustic detection (resonance), chemical analysis and laboratory examinations such as culturing, microscopy or serological tests (Clausen & Kartal 2003). However, culturing and microscopy are the only two methods considered to be definitive (Clausen & Kartal 2003) in evaluating wood colonisation by decay fungi.

Microscopic studies provide the baseline for evaluation of morphological changes in wood, for example, as observed for early decay also known as incipient decay (Wilcox 1993a) in *Abies* species for *O. placenta* and *G. trabeum* or in pinewood studying *O. placenta* and *C. puteana* (Irbe *et al.* 2006). Especially Wilcox's comparative light microscopy and scanning electron microscopy, examining early stages of brown rot decay, proved the two techniques were complimentary (Wilcox 1993a,b).

In this part of the PhD thesis, research is described of a microscopic study of laboratory-established fungal decayed *P. radiata*. The wood was inoculated with four isolated brown rot fungal strains, described in detail in Chapter 2, *S. lacrymans*, *A. sinuosa*, *O. placenta* and *G. trabeum*, and after twelve weeks of fungal colonisation, were microscopically studied specifically for wood/fungal interactions and degradation patterns.

The present PhD thesis research investigation aimed for correlating light microscopy (LM) and Field- emission scanning electron microscopy (FE-SEM) sequentially using the same 90 micrometer (µm) cut sections to detect and highlight micromorphological changes in moderate to advanced stages of decay. Further, it was sought to compare decay patterns between the four test fungi and correlate those findings to the respective mass losses from the decay tests, as described in Chapter 3.

5.2 Materials and Methods

For the microscopic investigation, preservative-free *P. radiata* blocks were used from the control sample range following the decay test as described in Chapter 3. Wood blocks were colonised for twelve weeks by one of the following four brown rot decay fungi chosen for the study:

- *Serpula lacrymans*
- *Antrodia sinuosa*
- *Oligoporus placenta*
- *Gloeopyllum trabeum*

5.2.1 Sample preparation

For every test fungus, one colonised *P. radiata* wood block was chosen of the ten replicates at the end of the decay test described in Chapter 3. Figure 5.1 shows all decayed wood blocks after the decay test were dried to equilibrium moisture content of 12% prior to microscopic examination. Blocks marked with a red star in the bottom right corner were chosen to determine any micromorphological changes compared to air dried and free from fungal infestation control *P. radiata* blocks. These control blocks were taken aside out of the same wood source as used for the decay tests (Chapter 3).

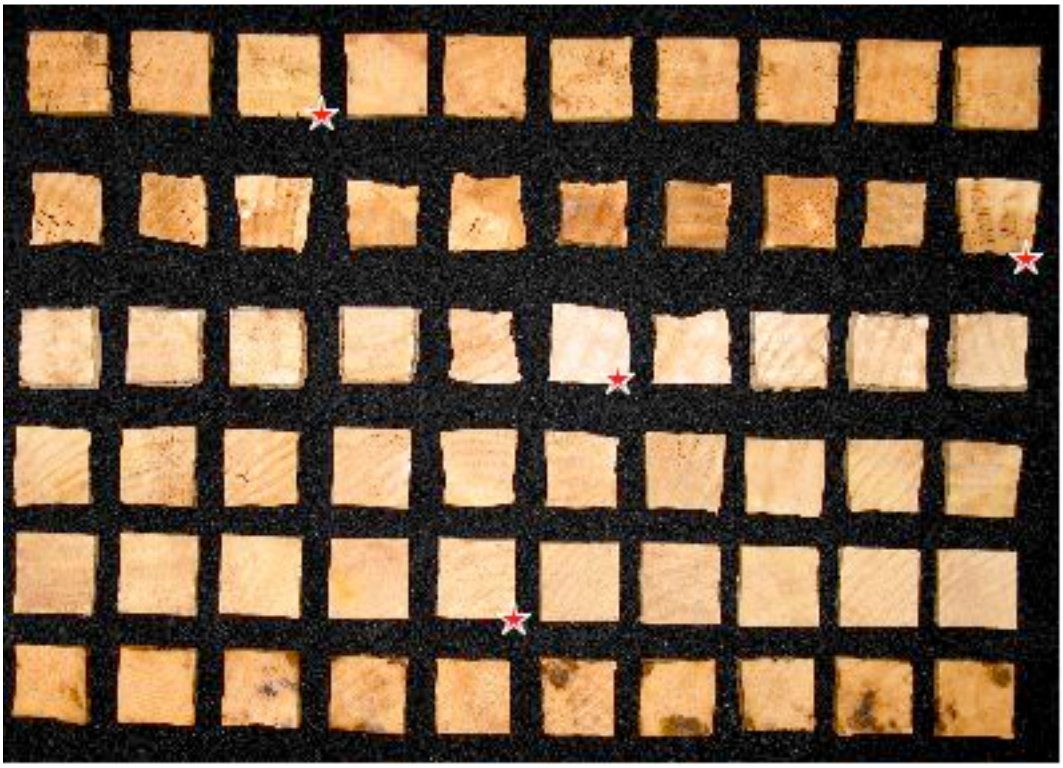


Figure 5.1 *P. radiata* after 12 weeks of fungal colonisation.
Top row: *Serpula lacrymans*; row 2: *Antrodia sinuosa*; row 3: *Oligoporus palcenta*; row 4: *Gloeophyllum sepiarium*; row 5: *Gloeophyllum trabeum* and row 6: *Coniophora puteana*.
Red stars highlight wood blocks examined microscopically.

The four marked wood blocks in Figure 5.1 and controls were soaked in distilled water for 30 seconds prior to cutting tangential and radial sections to a thickness of 90µm using a sliding microtome. To avoid cutting artefacts in transverse sections, it was found best to use a disposable hand-held microtome blade, aiming at thicknesses between 60 to 90µm. The thicknesses of radial/tangential and transverse sections were chosen to enable correlated microscopy, using the same sections for light microscopy (LM) and scanning electron microscopy (SEM). For each radial/tangential/transverse and wood/test fungus combinations, twelve sections were cut (n = 12) and examined to record the micromorphological changes.

Where possible, the majority of sections were taken from the edge or boundary of decay pockets within the blocks so that the interface of assumable early and advanced decayed wood cells could be examined. However, this was not achievable for all blocks/sections due to non-uniform decay patterns. Sections were only prepared from earlywood in radial and tangential directions for comparison of the different fungal-wood interactions. To study differences in the degradation of early- and latewood cells, those were best captured in transverse sections. An example of the orientation of sections cut is delineated in Figure 5.2. All sections were then stored immersed in distilled water in separate flasks with a screw top until further processing for microscopy and future reference.

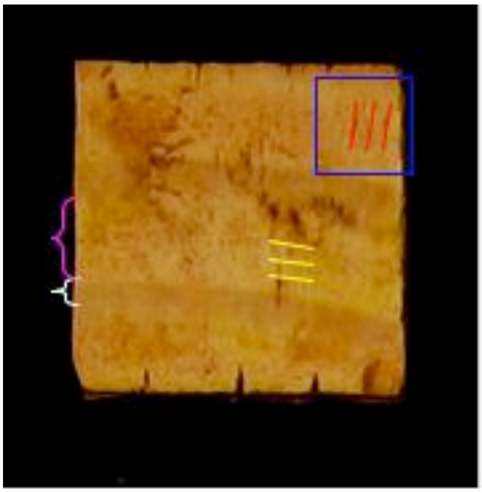


Figure 5.2 Test block after twelve weeks of colonisation by the brown rot fungus *Serpula lacrymans*.
Yellow lines: area of tangential cut sections; Red lines: area of radial cut sections;
Blue square: area of transverse cut sections; Pink brace: earlywood cells;
White brace: latewood cells.

5.2.2 Staining of wood sections

Specific staining techniques were employed to enhance and highlight selected features of interest contained in a wood section. Features such as fungal hyphal movement or degradation of pit membranes might otherwise not be revealed using LM. To collect this information, two different stains were used in this study, aqueous *Picro Aniline Blue* and aqueous *Toluidine Blue*.

5.2.2.1 Picro aniline blue stain

The stock solution was made by mixing 25ml saturated, aqueous aniline blue (Colour Index (C.I.) 42755) with 100ml saturated, aqueous picric acid.

Staining procedure:

Wood sections were immersed in distilled water under vacuum for ca. 30 min to cleave entrapped air. Afterwards, the sections were transferred (using a fine brush) into a new Petri dish containing 10ml of distilled water, to which was added 5 drops of Picro aniline blue stock solution. The sample was then either allowed to stand for at least 20 min or heated on a medium-warm hot plate (50-75°C) until the stain began to steam slightly. Finally, the section was washed at least two times in distilled water and mounted in 50% glycerin.

5.2.2.2 Toluidine blue stain

The stock solution was made by dissolving 0.1g Toluidine blue O (0.1%) (C.I. 52040) plus 1g Sodium tetra borate (1%) in 100ml distilled water.

Staining procedure:

Wood sections were immersed in distilled water under vacuum for ca. 30 min to cleave entrapped air. Sections were subsequently transferred using a fine brush into a new Petri dish containing distilled water diluted Toluidine blue stock solution (approx. 0.05%). The sections were allowed to stand for 2-3 minutes and then washed at least two times in distilled water. Finally, the sections were mounted in 50% glycerin.

5.2.3 Light microscopy

Wood sections were viewed under bright field, phase contrast and polarised light using a Zeiss Axioplan II light microscope equipped with 10X eyepiece lenses, 40X/0,90 oil/water/glycerol immersion Plan Neofluar and 20X/0,45 Achroplan lenses with an optovar intermediate tube for 1, 1.25, 1.6, 2 and 2.5X magnification. Results were captured with a mounted Zeiss AxioCam HRc digital colour camera and analysed with the manufacturer's imaging software AxioVision 40, Version 4.5.0.0. Scaling and measurements were produced with the same Zeiss software by prior calibration of each magnification setting using a stage micrometer slide, following the manufacturer's set-up guide. Further, measurement data form both LM and SEM pictures are given as a range and have been adjusted to the nearest accuracy limit of ± 0.5µm, due to the difficulty in setting the correct anchor points to an object of concern, which can depend on its clarity within the focal plane (Figure 5.5; hyphal measurement top left corner) and the observer's judgement. Cell wall thickness of earlywood cells were measured at the centre of two adjoining cells, recording the distance between the two S3 luminal layers.

5.2.4 Scanning Electron microscopy

Correlated microscopy was carried out with an electron microscope as it magnifies an object in a similar way to a light microscope. The difference lies in electrons instead of photons (light) being used and magnetic lenses rather than glass lenses achieving magnification. An electron microscope can operate in much higher resolution than the light microscope, providing additional topographic specimen information not detectable with the light microscope due to lights longer wavelengths compared to electrons. For example, the wavelength of visible light ranges between 200 to 750 nm (nanometer; Section 1.5.4), whereas the wavelength of an electron is 0.006 nm. The maximum resolution of an SEM is seen as 1 nm, or as sometimes expressed in spectroscopy 10 Ångström (Å).

In this PhD thesis research, a field emission scanning electron microscope (FE-SEM) 6700F manufactured by JEOL was used. Field emission (FE) describes the kind of electron emitter built into the microscope and this one was optimised for low accelerating voltage (0.5 – 15kV) making it suitable to observe non-conducting samples such as biological material.

Since biological material is non-conducting, it must be coated with, for example, chromium, carbon, gold or platinum to achieve conduction and stability of the sample concerned. In this study, wood sections were coated in an EMITECH K575X Turbo Sputter Coater with chromium as it provided a featureless surface even at the highest magnifications and more secondary electrons are produced compared to e.g. carbon coating. Chromium tends to oxidise rapidly and therefore samples were coated and examined within a few hours, usually within 3 to 5h.

Images were analysed and saved with the JOEL FE-SEM manufactures software, recording at the same time the microscope setting (Chapter 5.2.4.2) and corresponding magnification (scaling in pixels) as a text file. Scale bars were later produced using Digital Optics Ltd. V^{++} Precision Digital Imaging Systems software Version 4.0.

5.2.4.1 Scanning Electron mircroscopy sample preparation

In order for the wood sections examined by LM to be used for SEM analyses, sections needed to be dry, stable, conductive, non-magnetic and not out-gassing. This was achieved by the following protocol:

1. Removed the stained sections from the glass slides by immersion in distilled water in a Petri dish;
2. Soaked sections in 100% Ethanol for 1 minute;
3. Transferred sections onto a clean glass slide, covered with a second slide and used metal clamps to prevent curling of the sections;
4. Section left to air dry over night or placed clamped slides under vacuum for at least 30 minutes;
5. Applied carbon tapes to SEM stubs and centre-mounted wood sections; Note: the section had to be in good contact with the stub to avoid the build up of electrons on the surface of the sample (called charging) during microscopy;
6. Coated samples with chromium in sputter coater;
7. Loaded samples into FE-SEM for examination.

5.2.4.2 Microscope settings

Wood sections were examined using the following parameters:

- Accelerating voltage (AV)= 3kV
- Working distance (WD)= 15mm
- Probe current (PC)= 7
- Lower secondary electron detector= LEI or
- Upper secondary electron detector= SEI

5.3 Results and Discussion

For this investigation into micromorphological changes in fungal colonised *P. radiata,* light microscopy was firstly used, as it is especially suited for examination of many sections within a short timeframe, thus enabling a good pre-selection of sections for later correlated FE-SEM studies. Even though the chosen thickness of sections (60-90µm) may have prevented maximum resolution, they were still sufficient for distinguishing many diagnostic wood decay features Anagnost (1998). Complementary information was provided by FE-SEM while using the same wood sections as for LM (Figure 5.3), enabling higher resolution pictures detailing information about cell wall fractures. [Note that all the LM micrographs have the surname of the candidate in the field which was an identifying tool for the facility.]

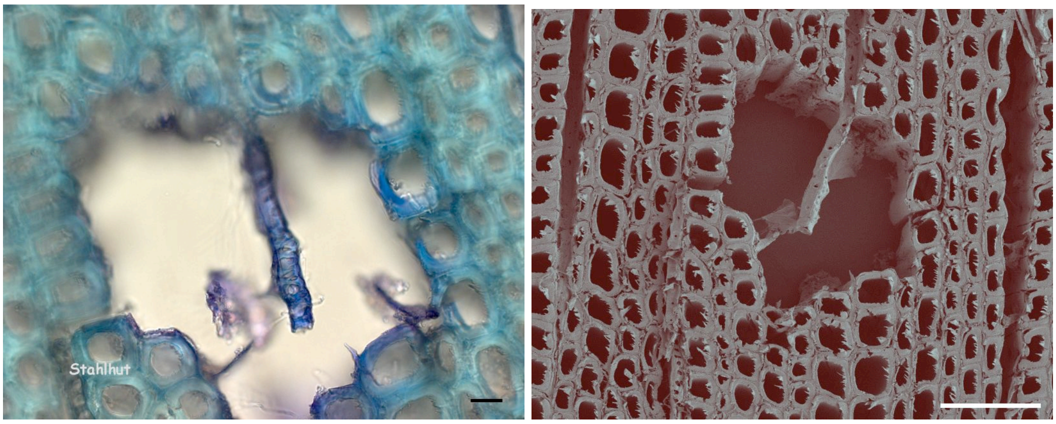


Figure 5.3 Correlated microscopic pictures of the same resin canal associated with a partly degraded wood ray. Wood sample was exposed to *Antrodia sinuosa*.
Left LM: Scale bar 10µm; right FE-SEM: Scale bar 100µm.

Although both instruments imaged most features, FE-SEM allowed for studies of the S3/S2 layer interface and S2/middle lamella (ML) region, details not readily brought into focus with LM. Similar observations were made by Wilcox (1993a) who concluded that especially small features such as individual hyphae, bore-holes and clamp connections were significantly easier to photograph by SEM. Micrographs of a chlamydospore of *O. placenta*, anchored into the S3 warty layer (Figure 5.4, and LM comparison Figure 5.5), a clamp connection (Figure 5.6) and a transverse section detailing S3/S2/ML cell walls (Figure 5.7), all pictured with FE-SEM, outlined that those features were difficult to bring into focus or detect using LM which highlights the advantages of using correlative microscopy. Additionally, measurements in FE-SEM micrographs can be regarded as more accurate (Wilcox 1993a), especially for thin hyphal diameter as seen in Figure 5.7, an *O. placenta* hyphae measuring 1.3µm.

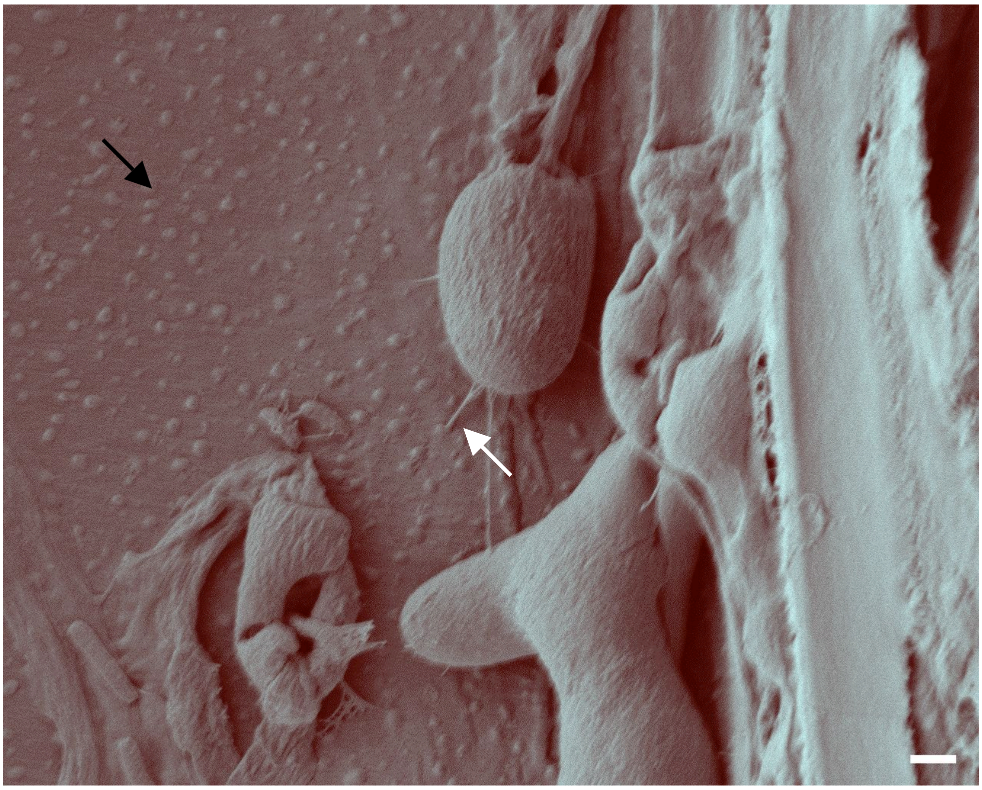


Figure 5.4 *Oligoporus placenta* chlamydospore anchored to the warty/S3 layer of the tracheid (white arrow) and showing warts on the lumen surface (black arrow). Scale bar 1µm.

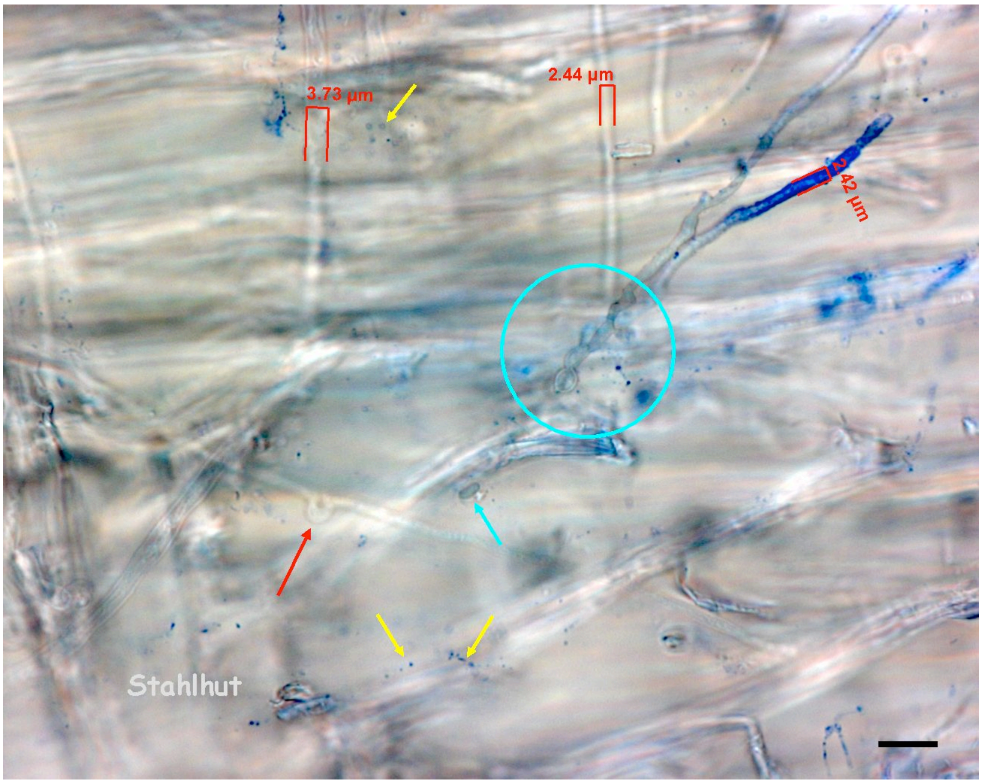


Figure 5.5 *Oligoporus placenta* chlamydospore formation (turqousie circle) and deposition (turquoise arrow), clamp connection (red arrow), slime/mucilage surrounding hyphae (yellow arrows) and measurements of hyphae. Scale bar 10µm.

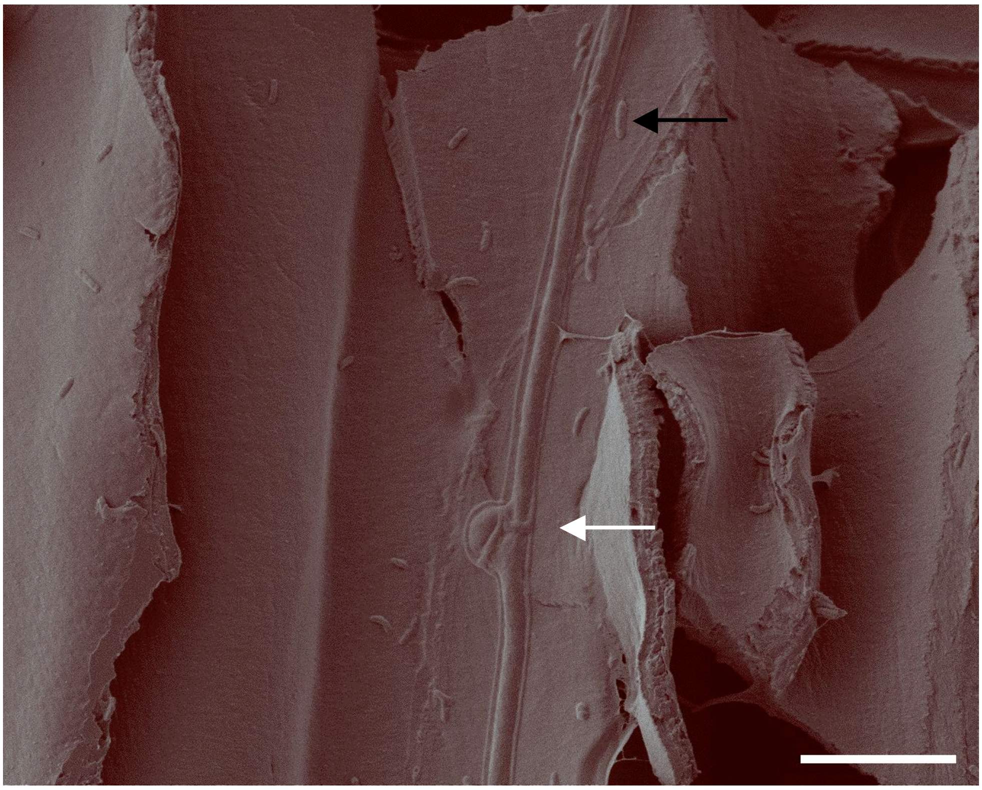


Figure 5.6 Clamp connection of *Oligoporus placenta* (white arrow) and bacteria (black arrow). Scale bar 10µm.

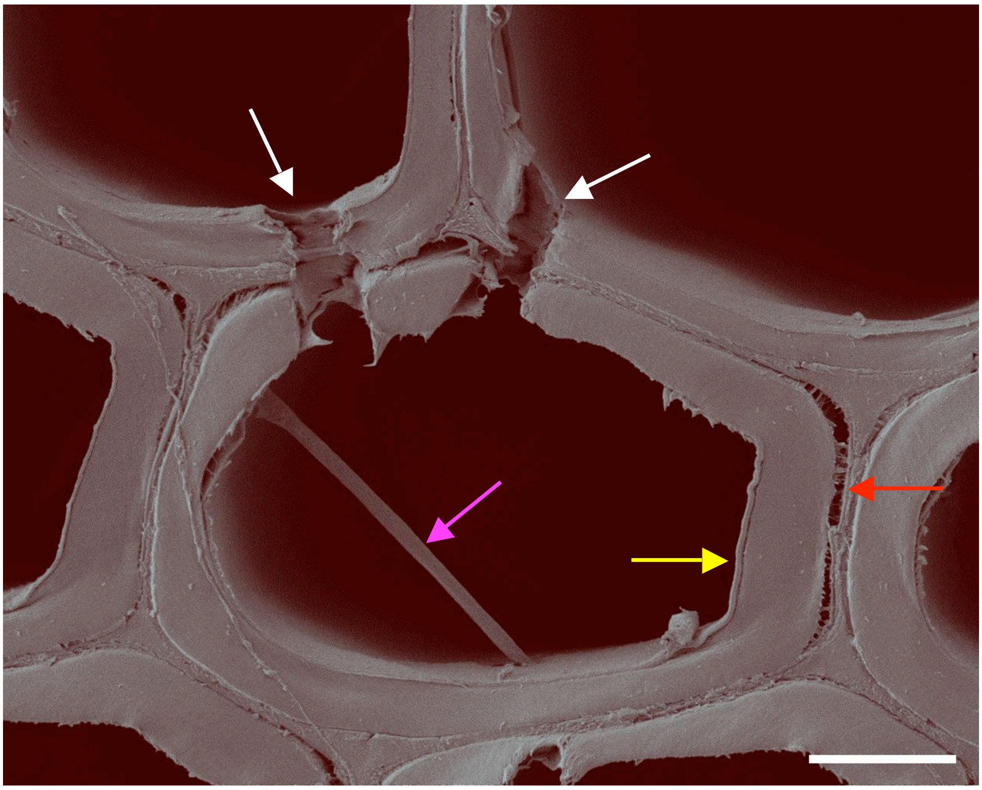


Figure 5.7 Transverse section showing hypha in lumena (pink arrow), bore-holes (white arrows), an intact S3 layer (yellow arrow) and the start of S1 delamination at the middle lamella/S1 interface (red arrow). Scale bar 10µm.

A recent study by Singh *et al.* (2008) using correlated light, confocal and scanning electron microscopy of wood-adhesive interfaces found the light microscope unable to bring particularly the distant wood tissues in the same focal plane as the wood-adhesive. Knowing this limitation of the LM, this PhD thesis research tried to compensate by photographing the area of interest several times over several focal planes, but under the same magnification to not alter the scaling. Photo editing software does allow for the insertion of one picture into the other (layering of pictures), enabling the presentation of many e.g. decay features in an 'one focal plane picture' without altering the originals. Figure 5.8 shows a growing clamp connection (yellow arrow), originally photographed in a different focal plane than the ordinary clamp plus a clamp at a T-branch (red arrows). A second example of layering two pictures to enhance presentation is given in Figure 5.15, showing the early- and late- wood interface of a transverse section together in focus.

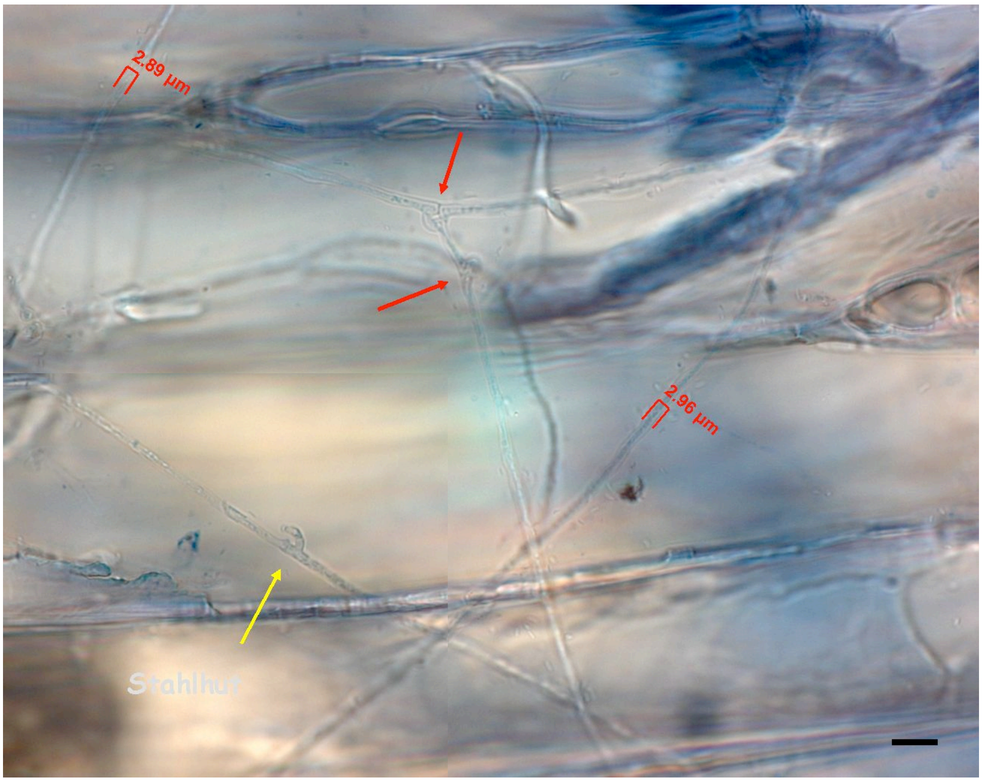


Figure 5.8 *Oligoporus placenta* hyphae pictured in different focal planes and layered to enhance presentation of clamp connection features. Initiation of clamp formation (yellow arrow) and two ordinary clamps, one thereof at a T-branch (red arrows) and measurements of hyphae. Scale bar 10µm.

5.3.1 Pinus radiata control sections

P. radiata control sections that were examined with LM and subsequently FE-SEM, clearly showed intact tracheids, ray parenchyma cells, resin canals and intact, but mainly closed, bordered and simple cross-field pit membranes (Figures 5.9 to 5.14). The latter is a natural phenomenon often occurring during the drying process where air replaces water in the wood cells. Here, pit membranes became aspirated with the result of the membrane closing either side of the pit apertures (Burnes *et al.* 2000). Not only does this end the water permeability of the pit chamber but is also a hindrance for fungi or bacteria mobility between cells. Further, all naturally air dried (ca. 12% MC) control blocks sections examined showed no signs of sapstain, mould, decay fungal or bacterial pre-infection; these have been shown to be likely to occur in the wood yard before processed lumber were dried to approximately 18% MC of its dry mass (Wakeling *et al.* 2000). Average Cell wall thickness was recorded with 7.4 ± 1.5 µm (Appendix 4).

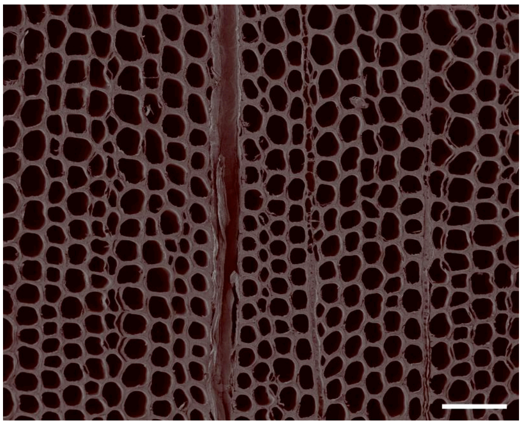


Figure 5.9 Transverse section of control block showing intact earlywood cells and rays. Scale bar 100µm.

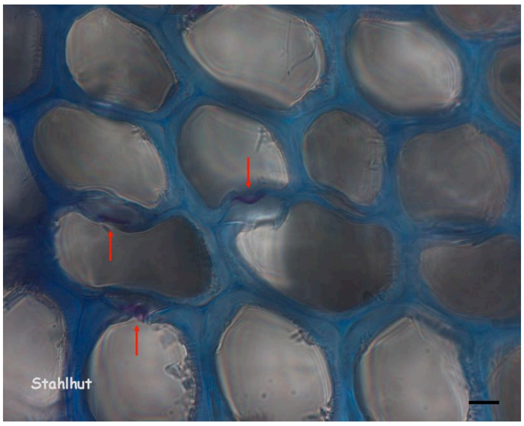


Figure 5.10 Toluidine blue stained earlywood control block cells showing closed, but intact pit membranes (red arrows). Scale bar 10µm.

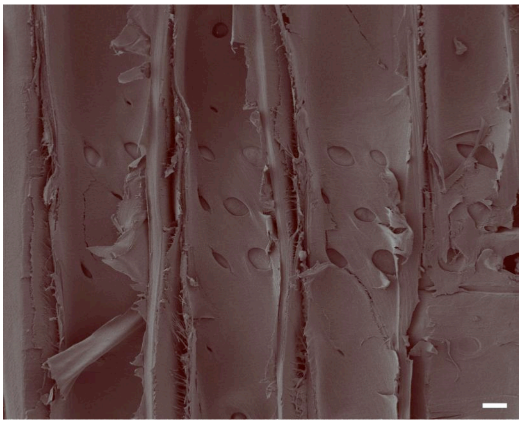


Figure 5.11 Radial section of control block showing intact, but closed simple cross-field pits. Scale bar 10µm.

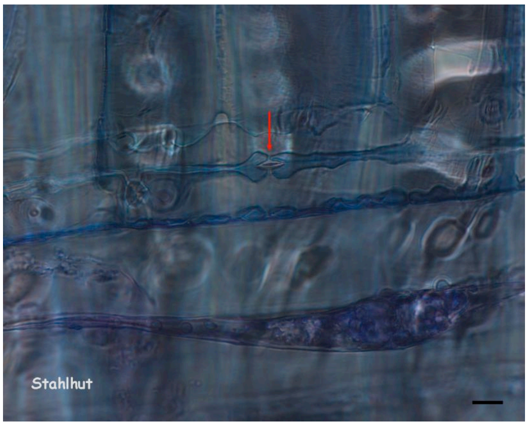


Figure 5.12 Wood ray of control block showing intact simple pits (red arrow). Scale bar 10µm.

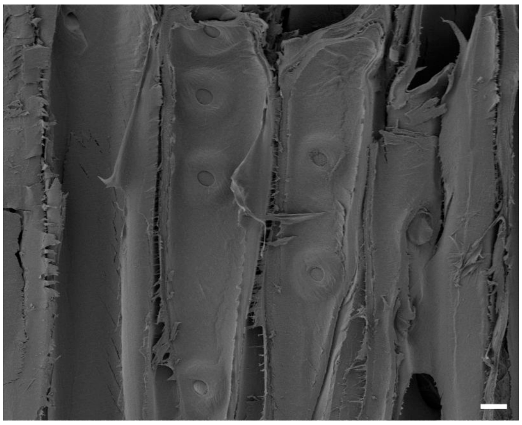


Figure 5.13 Tracheids in control block showing intact, but closed bordered pit membranes. Scale bar 10µm.

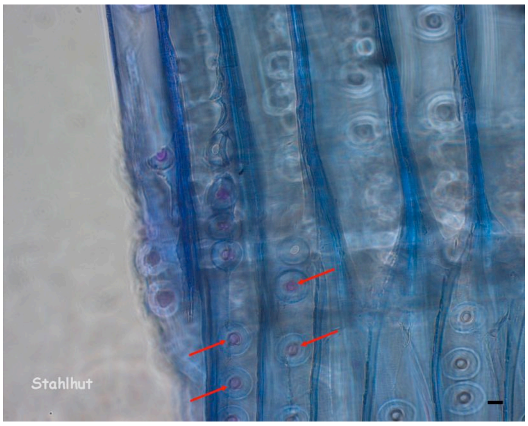


Figure 5.14 Tracheids in control block stained with toluidine blue showing intact, but closed bordered pit membranes (red arrows). Scale bar 10µm.

Staining sections with Toluidine blue (control sections and transverse sections of decay test specimens) proved effective in clearly differentiating early- and late-wood cells, the middle lamellae and pit membranes. Toluidine blue is a metachromatic dye which stains lignified cell walls green or greenish-blue, whereas primary cell walls (i.e. epitheral cells in resin canals or parenchyma cells in rays; Figure 5.3) and pit membranes stain pinkish-purple because of their polyuronide (such as pectin) content (O'Brien & McCully 1969). As can be seen in Figures 5.10 and 5.14, the pit membranes stained purple highlighting those membranes sealing either side of the pit apertures (transverse section) or the upper pit apertures within lens focus (radial section).

Since Toluidine blue reacts preferentially with, and stains lignin within the cell wall, it imparts a dark blue colour to the middle lamellae, a light green-blue on latewood cells and darker greenish-blue on earlywood cells, i.e. the greater the concentration of lignin the greater the intensity of the blue colour reaction (Figure 5.15).

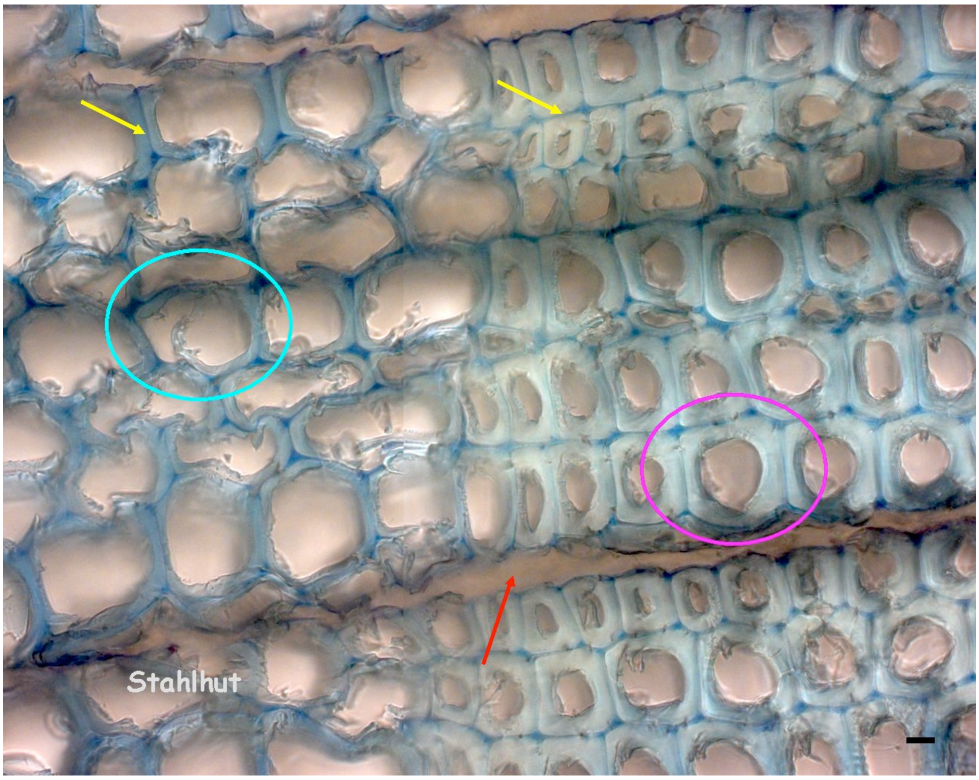


Figure 5.15 Toluidine blue stained transverse section showing degraded cells after 12 weeks of colonisation by *Gloeophyllum trabeum*. Early- and late- wood are photographed in two focal planes and layered. Middle lamellae stained dark blue (yellow arrows), degraded ray (red arrow), light green-blue intact latewood cells (purple oval) and darker greenish-blue distorted or broken earlywood cells (turquoise oval and surroundings). Scale bar 10µm.

Wood cells with a thinner S2 layer, and which appeared to have a lower cellulose content (either due to degradation or defects during cell formation), were stained darker (Figure 5.16).

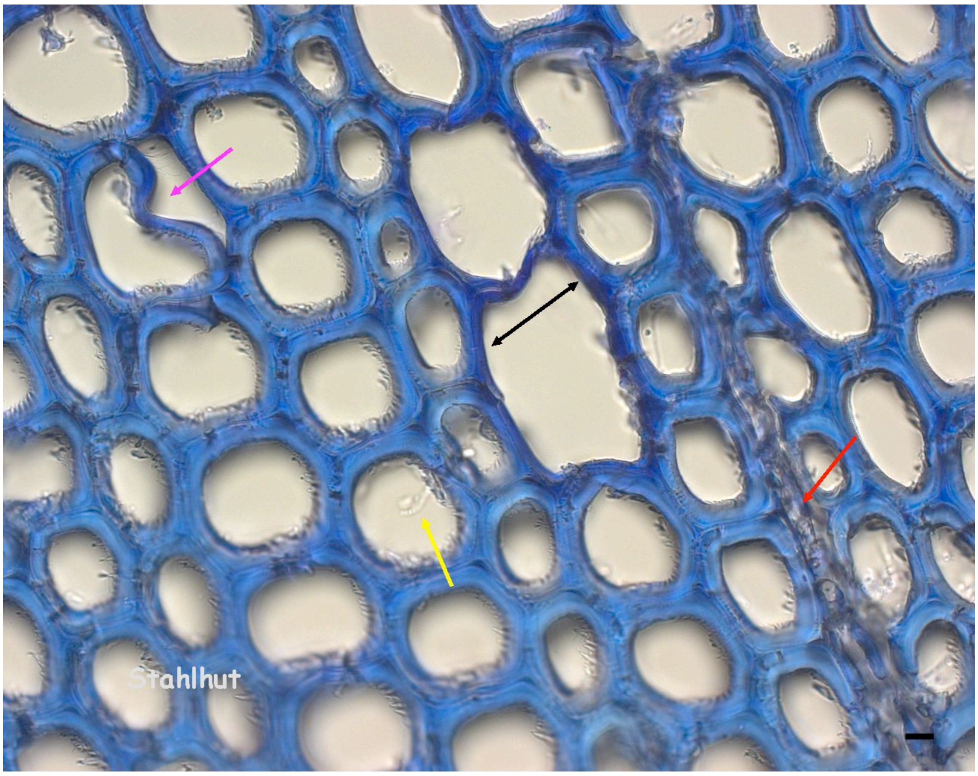


Figure 5.16 Toluidine blue stained section from wood after 12 weeks of colonisation by *Antrodia sinuosa,* showing a thinned S2 layer (black arrow), intact wood ray (red arrow), cell wall buckling (pink arrow) and a hypha penetrating through a bordered pit (yellow arrow). Scale bar 10µm.

5.3.2 Microscopic observations for the interaction between test fungi and Pinus radiata

The Picro aniline blue stain proved effective in differentiating growing fungal hyphae from the wood cells, highlighting fungal features such as clamp connections and chlamydospores (Figures 5.18 to 5.21) and detecting bacteria throughout some tracheids and rays, the latter only shown in FE-SEM Figure 5.6. Following the protocol in Section 5.2.2.1, fungal hyphae and bacterial cells were easily identified as they appeared blue under the LM; the dye was originally developed by Cartwright (1929), and later modified by Wilcox (1964) to highlight and differentiate fungal hyphae in wood and it also successfully to revealed bacteria in wood (Knuth 1964). However, as found with this investigation, some wood cells appeared slightly bluish in *P. radiata* (Figure 5.13), diminishing contrast between fungal and wood features. This was a particular problem for the close relationship between the hyphae attached to the S3/warty lumenae layer. This was speculated to be due to insufficient destaining in distilled water, rather than actual uptake by the wood cell wall.

Table 5.1 summarises observations made with correlated microscopies for the four brown rot test decay fungi.

Table 5.1 Summary of micromorphology of brown rot fungi on *P. radiata* ‘decay test’ blocks.

Fungus	**State of anatomical wood features**				**State of cells in**		**Fungal hyphae**			**Bore holes**		**Clamps**		**Chlamydo-/ Arthro-spores**	**Oxalate crystals**	**Slime layer**	**Weight loss % mean (range)**
	Ray parenchyma	Middle lamellae	Pit membranes B	Pit membranes R	Earlywood	Latewood	Position in lumen	Size (µm)	Frequency	Present	Size (µm)	Ordinary	Medallion				
Serpula lacrymans	little change	no change	✗	✗	S2-PW delamination	no change	random	1-4	≤1/tracheid ≥5/ray	$\checkmark^{18}$	2-2.5	$\checkmark^{18}$	✗	$\checkmark^{20,21}$	✗	✗	11 (14-8)
Antrodia sinuosa	largely missing	no change	✗	✓	S2-PW delamination, thinning, fractures	little cell wall distortion	random	1-3.5	≤3/tracheid ≤3/ray	$\checkmark^{25}$	1-5	$\checkmark^{26}$	$\checkmark^{26}$	$\checkmark^{24}$	✗	✓	55 (68-36)
Oligoporus placenta	largely missing	no change	$\checkmark^{29}$	✗	S2-PW delamination, fractures	little cell wall distortion	random	1-3.5	≥5/tracheid ≤3/ray	$\checkmark^{36}$	2-3.5	$\checkmark^{6}$	$\checkmark^{38}$	$\checkmark^{4,5,37}$	✗	✓	35 (51-27)
Gloeophyllum trabeum	largely missing	no change	✗	✗	S2-PW delamination, fractures	no change	random	1-5	≥5/tracheid ≥5/ray	$\checkmark^{43,44}$	1-4.5	✓	✗	$\checkmark^{47}$	✗	✓	15 (22-6)

$\checkmark^{(1)}$= Feature present; superscript number references to corresponding Figure in Chapter 6 (i.e. $\checkmark^{29}$ = Fig 6.29);
✗= Feature absent/not detected;
S2= Inner secondary cell wall layer;
S3= Secondary cell wall layer including the warty layer (W);
PW= Primary cell wall layer including secondary cell wall layer S1 and the middle lamella;
LW= Latewood;
B= Bordered pit;
R= Ray/crossfield pit;
Random= Hyphae attached to the lumen layer and throughout the lumen.

5.3.2.1 Observations for *Serpula lacrymans* colonised wood blocks

Macroscopically, the *S. lacrymans* colonised test blocks showed early signs of degradation, seen as decay pockets, mainly localised in the sapwood, and splitting of cells in the outer regions (Figure 5.1 and 5.2). The respective mass loss recorded for the test block of this investigation was minor at 11%, which led to only minor shrinkage after drying to 12% MC.

Light- and complementary scanning electron microscopic observations revealed that most of the fungal activity was within the rays, and fewer hyphae were observed within earlywood cells. Even though colonisation of ray parenchyma was advanced, much of the ray parenchyma was still intact (Figures 5.19 and 5.22). Furthermore, the latewood cells examined showed no fractures of the cell walls. Earlywood cells on the other hand showed partly restricted advanced cell wall fractures such as buckling (S2 layer advances into the cell lumen; Figure 5.17) or early delamination/separation at the S2/ML interface as illustrated in Figure 5.7. Middle lamellae and S3 layer were found intact in both earlywood and latewood. Earlywood cell wall thickness was recorded as unchanged with 7.7 ± 1.5 µm.

S. lacrymans produced a few fine bore-holes for cell migration (Figure 5.18), with the smallest and the biggest diameter recorded at 2µm and 2.5µm respectively. Hyphal size varied between 1µm and 4µm, and where still present, had about the same size diameter as the corresponding bore-hole. Pit membranes, both bordered and simple, were found to be degraded to a great extent.

Two other growth features detected were the deposition of thin-walled and cylindrical arthrospores located in axial tracheids with a maximum size of 2.5x5µm (Figures 5.20 and 5.21) and ordinary clamp connections (Figure 5.18).

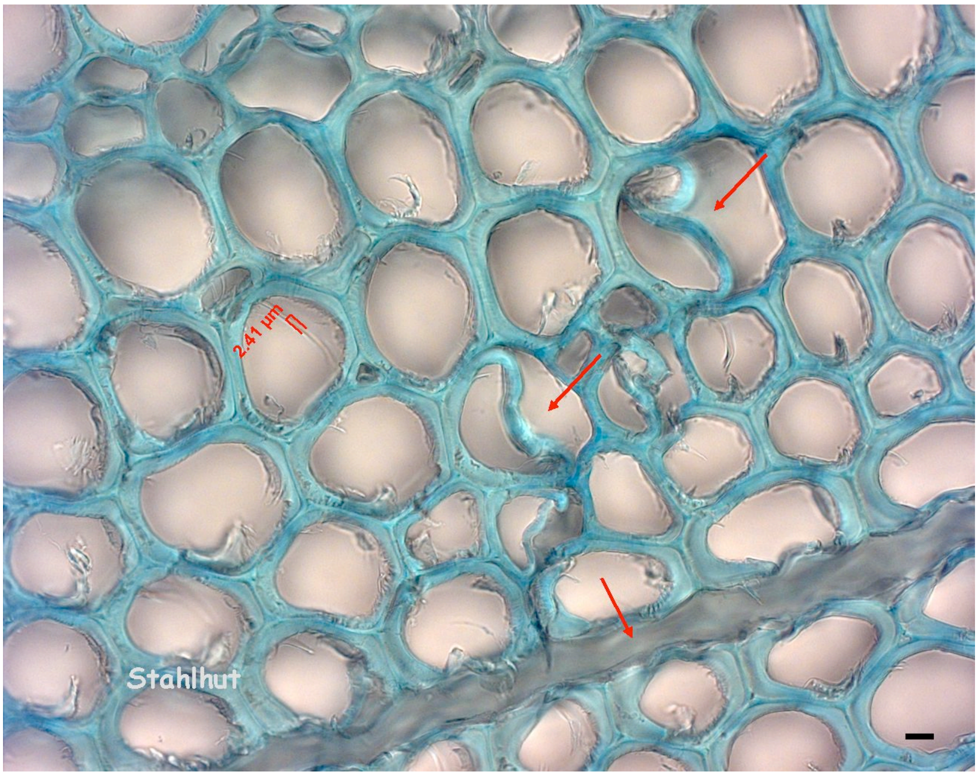


Figure 5.17 Toluidine blue stained transverse section showing cell buckling and separation (red arrows) and *Serpula lacrymans* hyphal measurement. Scale bar 10µm.

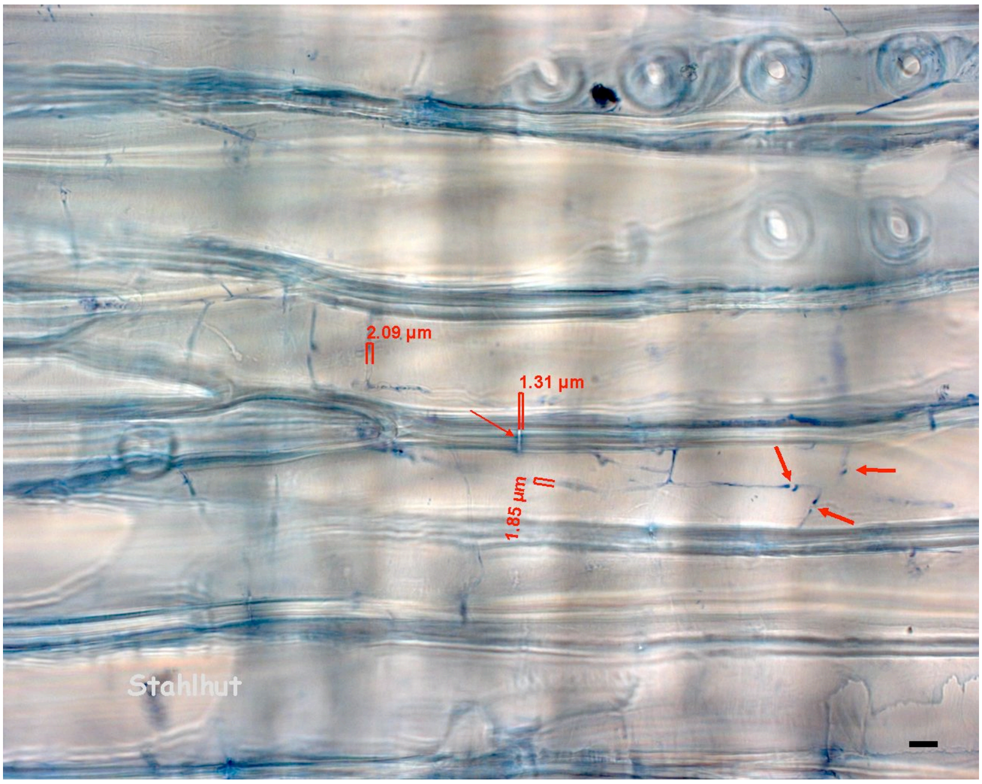


Figure 5.18 Picro aniline blue stained hyphae of *Serpula lacrymans* in radial section showing clamp connections (bold red arrows), hyphal measurement, and bore-hole measurement (thin red arrow). Scale bar 10µm.

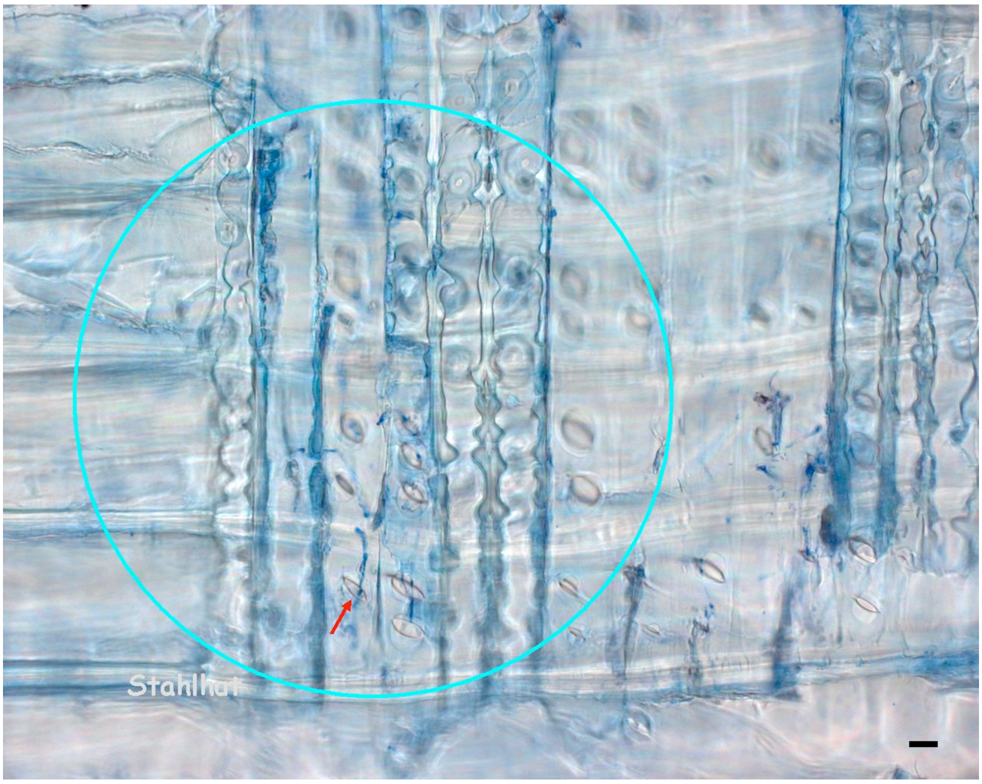


Figure 5.19 Partly intact wood-ray/tracheid cross-field (turquoise circle) in radial section with *Serpula lacrymans* hypha located between ray and tracheid simple pit (red arrow). Scale bar 10µm.

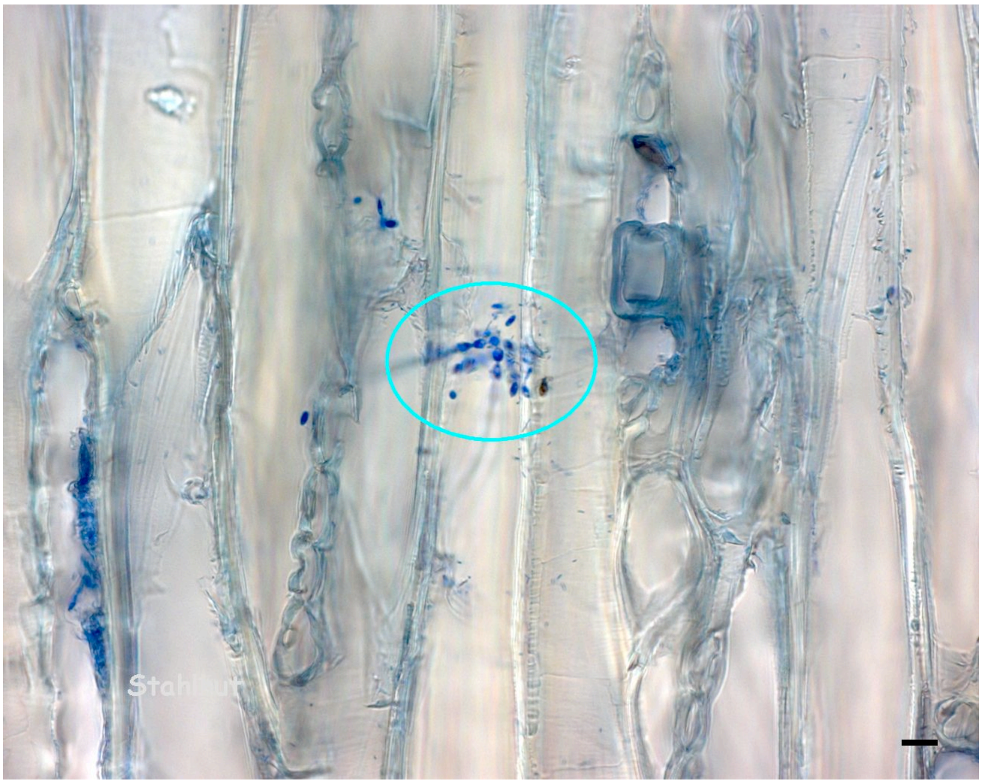


Figure 5.20 Arthrospores of *Serpula lacrymans* deposited in axial tracheid (turqoise oval) in tangential section. Scale bar 10µm.

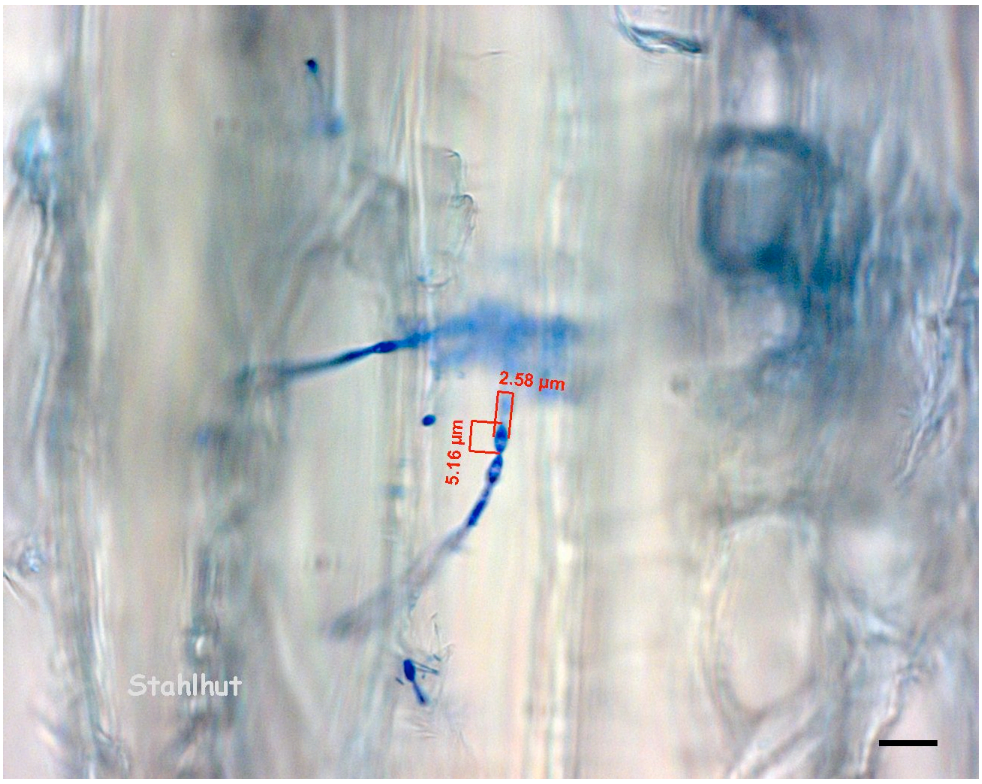


Figure 5.21 *Serpula lacrymans* arthrospore formation and measurement in tangential section. Scale bar 10µm.

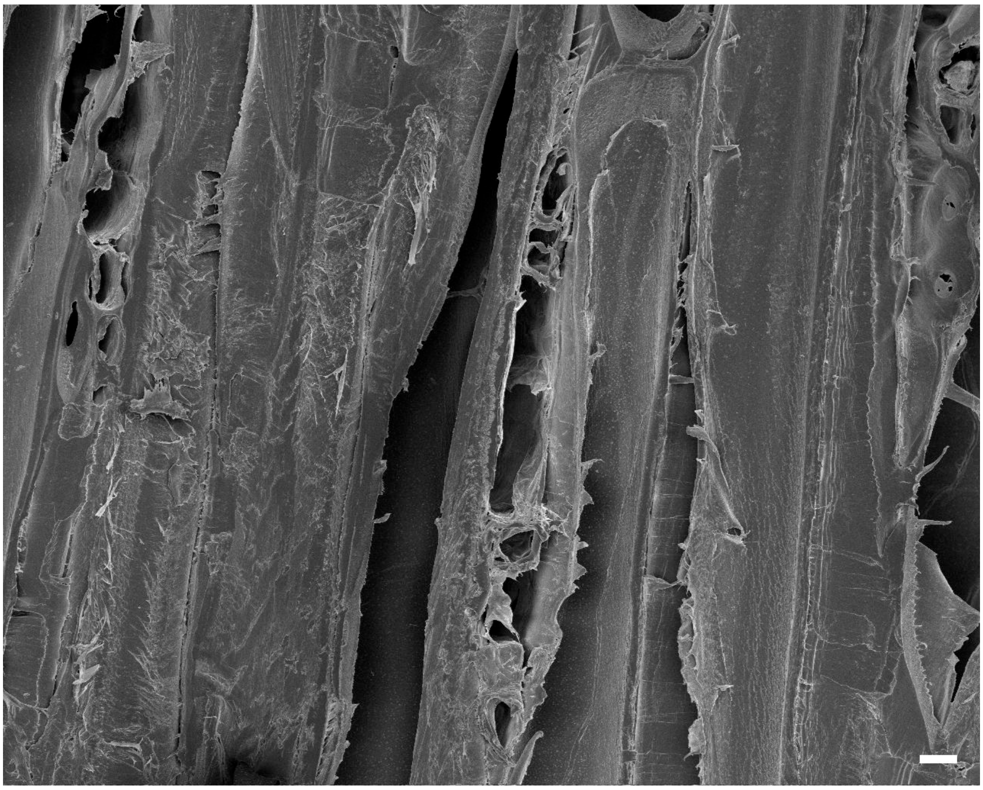


Figure 5.22 Partly degraded cells in a ray in tangential section. Scale bar 10µm.

5.3.2.2 Observations for *Antrodia sinuosa* colonised wood blocks

Test blocks colonised by *A. sinuosa* macroscopically showed severe signs of degradation as only small areas of the original wood structure could be detected (Figure 5.1) and this was consistent with the 36% mass loss recorded for the test blocks, resulting in severe shrinkage at 12% MC; many cracks formed within the earlywood and blocks fractured easily when handled.

Observations with correlated microscopy found fungal hyphae branching within every cell and wood rays (at the interface between heavily degraded to more sound wood cells), with the rays, compared to *S. lacrymans*, much more degraded and only a few partly intact (Figure 5.24). Latewood cells showed some distortion, whereas earlywood cells showed all signs of trans-wall fractures from separation, distortion, thinning, buckling to cell breakage (Figures 5.23 and 5.27). Comparing those fractures to the other three decay fungi, *A. sinuosa* was the only brown rot where a thinning of the S2 earlywood cell wall layer was observed (Figure 5.16) with average thickness of 6.4 ± 1.5 µm (Appendix 4). Pit membranes of both types were usually degraded, however, even at this advanced stage of decay a few membranes in the ray pit field were still intact. Middle lamellae and S3 layer remained intact in both early- and late- wood.

Bore-holes produced by *A. sinuosa* were sparsely distributed over the sections examined, however with minimum and maximum sizes of 1µm to 4µm respectively, it showed the greatest variability of all four test fungi (Figure 5.25). Bore-hole and hyphal size stood in close relationship, with the hyphae having the same diameter. Their size varied between 1µm and 3.5µm. In some tracheids a slime/mucilage layer surrounded thin hyphae (Figure 5.53 Section 5.3.3), a feature not detected for *S. lacrymans* sections.

As for *S. lacrymans*, *A. sinuosa* produced chlamydospores and clamp connections (Figure 5.25). Chlamydospores appeared round, most often deposited in wood rays and varied in size from 2.5µm to 9.5µm. Ordinary clamp connections were observed frequently with the addition of some medallion clamps being formed in some tracheids (Figure 5.26).

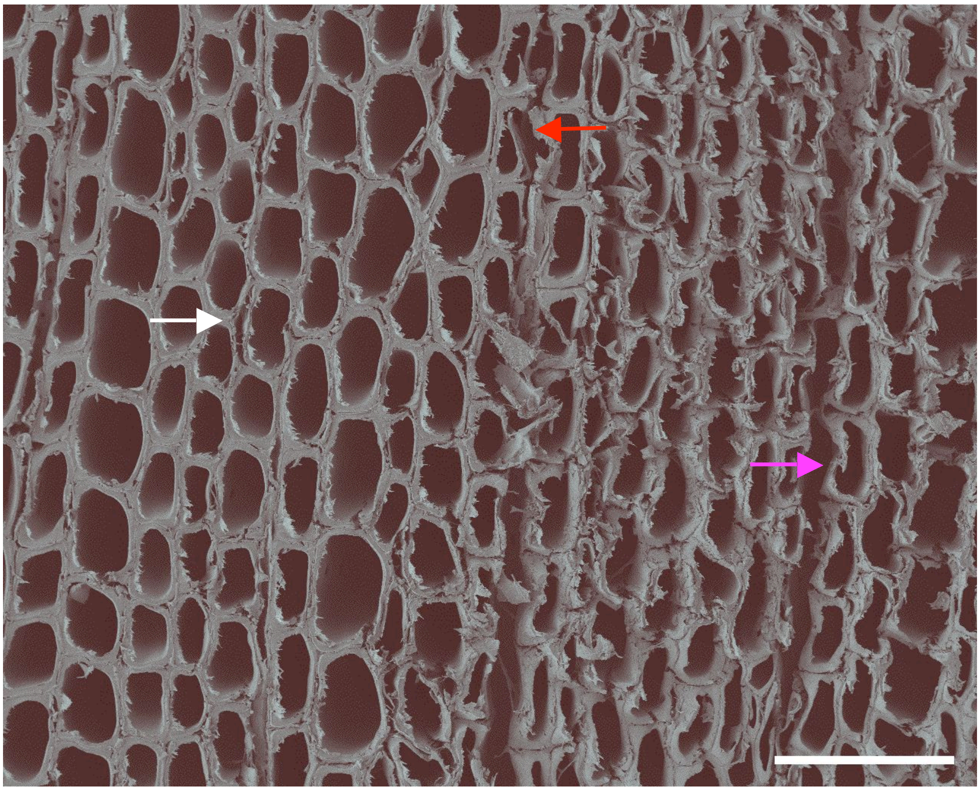


Figure 5.23 Transverse section showing progressive trans-wall fractures from the left to the right, caused by *Antrodia sinuosa*. Cell separation/delamination of S2 in intact cells (white arrow), cell buckling and breakage (red arrow) and distortion (pink arrow). Scale bar 100µm.

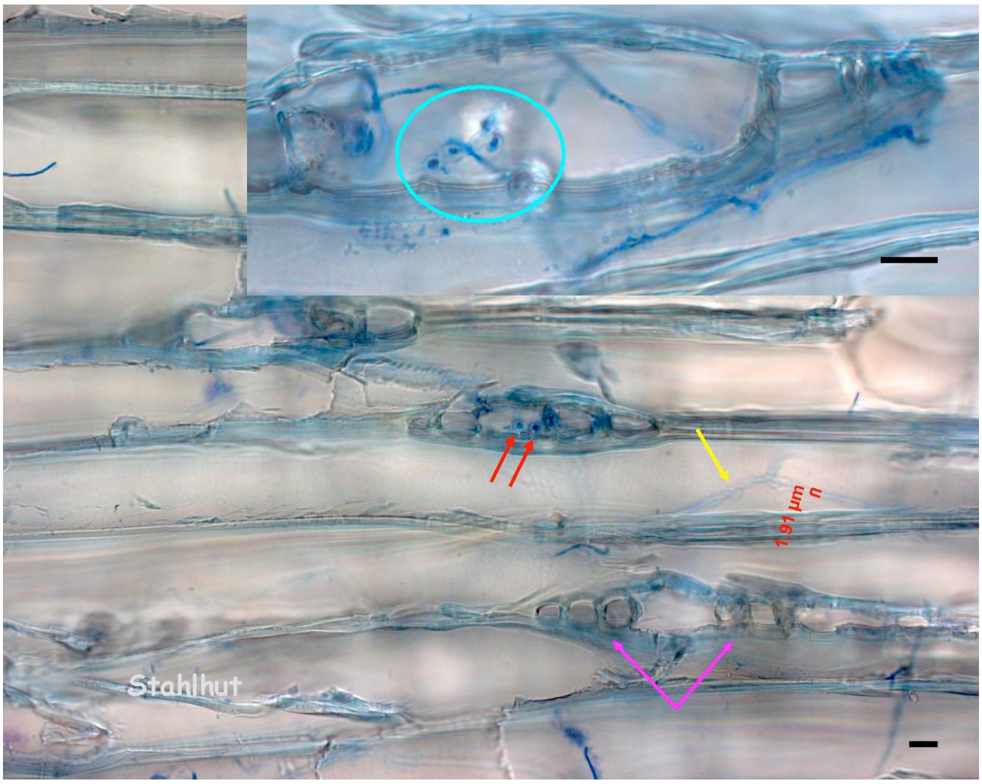


Figure 5.24 Tangential view showing *Antrodia sinuosa* chlamydospores (red arrows and turquoise oval in close up view in the inset), ordinary clamp connection (yellow arrow), in parts intact ray parenchyma cells (pink arrows) and hyphal measurement. Scale bars 10µm.

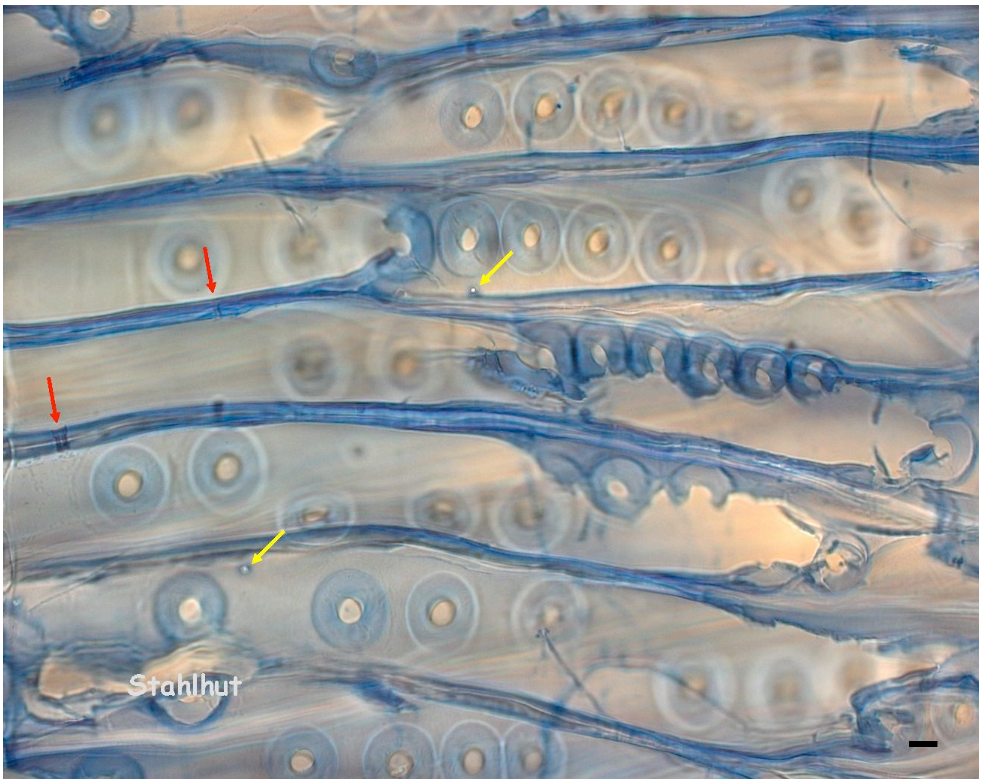


Figure 5.25 Radial section showing bore-holes produced by *Antrodia sinuosa*. Bore-holes seen as a tunnel through the cell wall (red arrows) and from the top (yellow arrows). Scale bar 10µm.

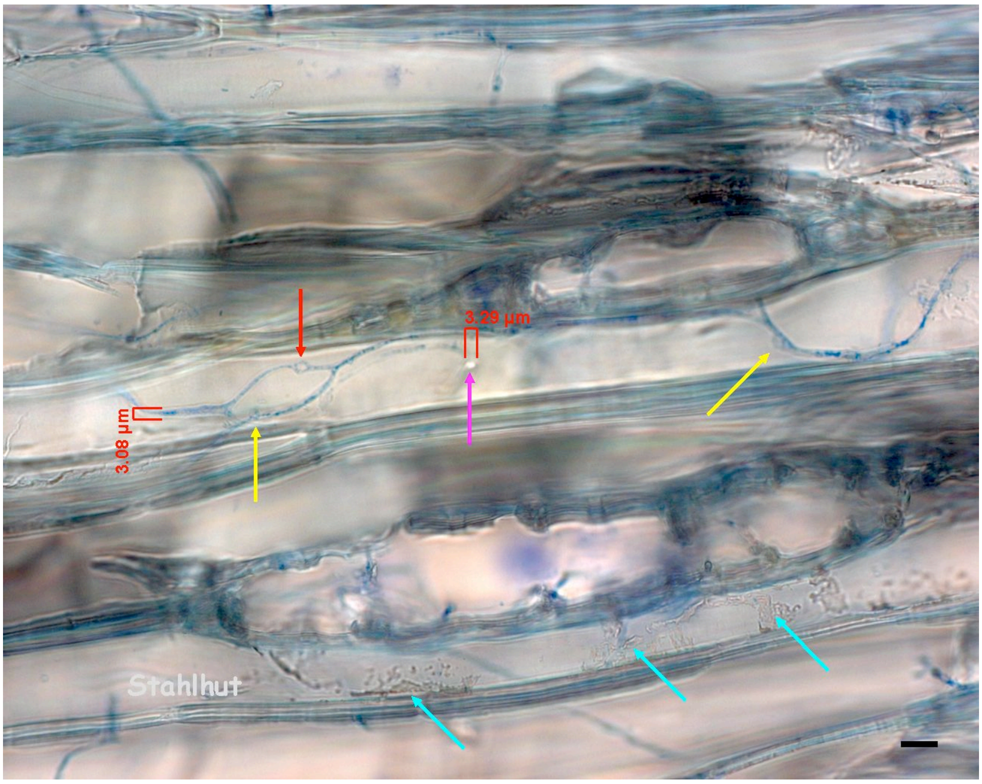


Figure 5.26 Tangential section showing ordinary clamp connections (yellow arrows), medallion clamp (red arrow), bore-hole (pink arrow), cell wall fracturing (turquoise arrows) and bore-hole and hypha measurements. Scale bar 10µm.

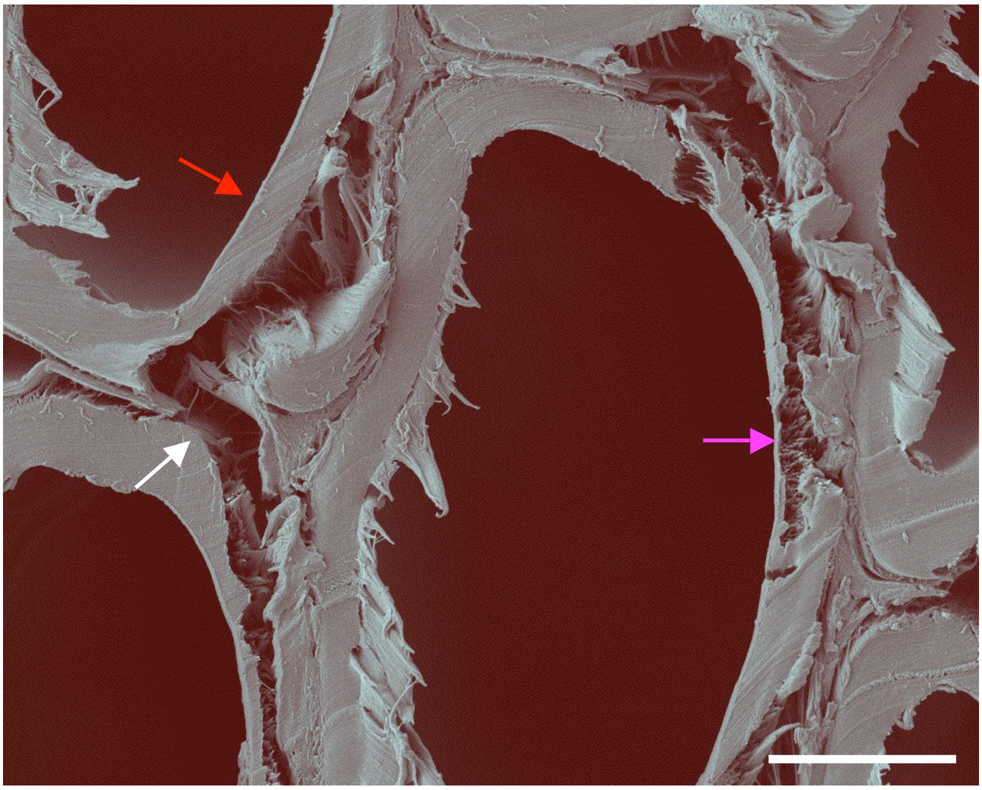


Figure 5.27 Degradation of the S2 cell wall layer by *Antrodia sinuosa* showing cell separation and breakage at the middle lamella interface (white arrow), intact S3 layer (red arrow) and delaminated microfibres in the S2 layer (pink arrow) in transverse view. Scale bar 10μm.

5.3.2.3 Observations for *Oligoporus placenta* colonised wood blocks

Advanced stages of degradation, most often in pockets throughout earlywood, were observed for *O. placenta* test blocks (Figure 5.28). Mass loss after 12 weeks was 33%, very similar to the mass loss result of *A. sinuosa*, but macroscopically no block internal cracks and cell splitting could be seen, hence a visually more intact surface picture. However, when photographed after drying to approximately 12% MC, severe shrinkage had occurred, comparable to *A. sinuosa* degraded blocks (Figure 5.1).

Correlated microscopy revealed many hyphae growing and branching within the lumenae of earlywood tracheids and less in the remaining ray parenchyma cells (Figure 5.30). Hyphal size varied between 1μm for very thin to 3.5μm for maximum diameter. Latewood cells had been colonised resulting in distortion of some cells or early trans-wall fractures. That latewood degradation was at an early stage could best be determined in transverse sections, showing cell shape and orientation plus the presence of bordered pit membranes in some of the sections (Figure 5.29). Except of cell wall thinning, as was only detected in *A. sinuosa* degraded blocks, all advanced degradative patterns in earlywood cells were observed. Average cell wall thickness was recorded with 7.6 ± 1.5 µm. Figures 5.31 to 5.33 present some of the trans-wall fractures such as cell delamination, distortion, buckling and breakage, while pictures were taken at either side and on the borderline between early to advanced stages of brown rot decay.

Even though most of the test blocks were found in an advanced degraded state some wood rays still contained undegraded parenchyma cells (Figure 5.34) and intact pit membranes (Figures 5.29 and 5.35). For the middle lamellae and S3 lumenae layers, both were found intact (Figure 5.7).

O. placenta degraded sections showed many bore-holes which were best captured with tangential cuts (Figure 5.36), with the hyphae penetrating through them being smaller in diameter. The smallest bore-hole diameter detected was 2µm in size and the biggest recorded was 3.5µm, which is slightly smaller than the widest hyphae measured.

As presented in Figures 5.4 and 5.5, *O. placenta* produced round to oval shaped chlamydospores (Figure 5.37) similar to the arthrospores of *S. lacrymans*, but with maximum dimensions of 4.5 x 5µm, not as elongated. Deposition of these spores was found to be in the axial tracheids.

Many ordinary and medallion clamp connections were formed by *O. placenta*, together with *A. sinuosa* the only two fungi observed to form both types (Figures 5.6 and 5.38). Additionally, and only observed for *O. placenta* was the ordinary clamp formation directly connected with the fungus branching into a second direction by initiation of a T-branch (Figure 5.8).

In some radial or tangential sections a thin slime/mucilage layer occurred surrounding the hyphae (Figure 5.5), similar to the layer detected in *A. sinuosa* samples.

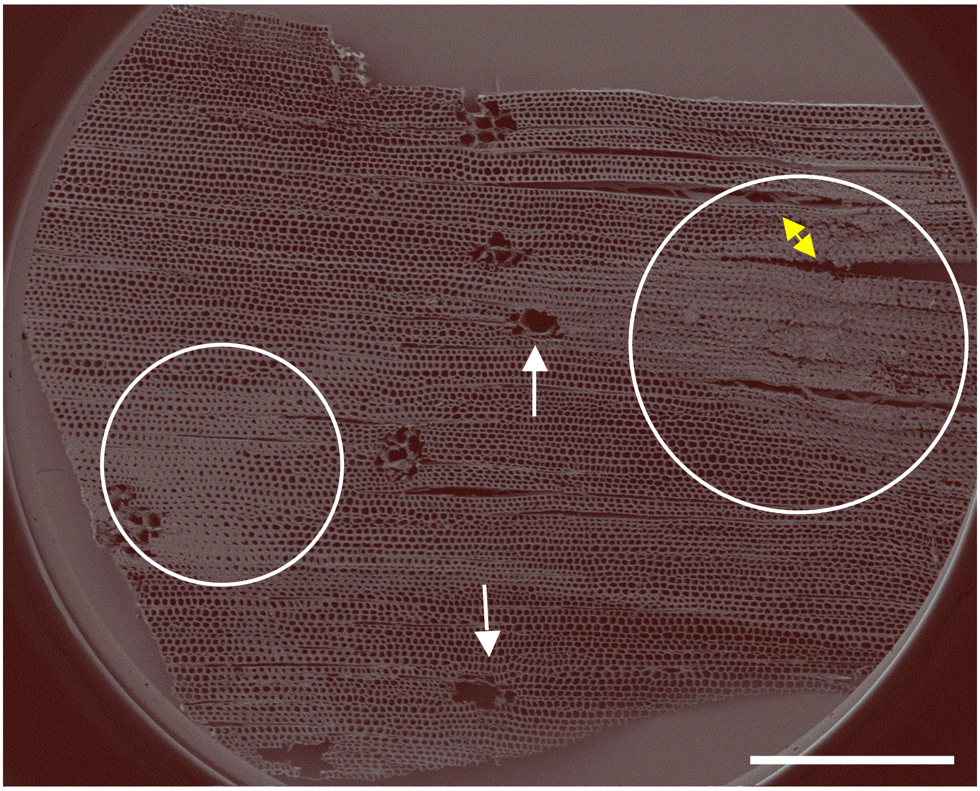


Figure 5.28 Topographical view of *Oligoporus placenta* degraded section under low magnification FE-SEM. Partly degraded resin canals (white arrows), cells separation/splitting (yellow arrows) and pockets of heavily degraded cells (white circles). Scale bar 1mm.

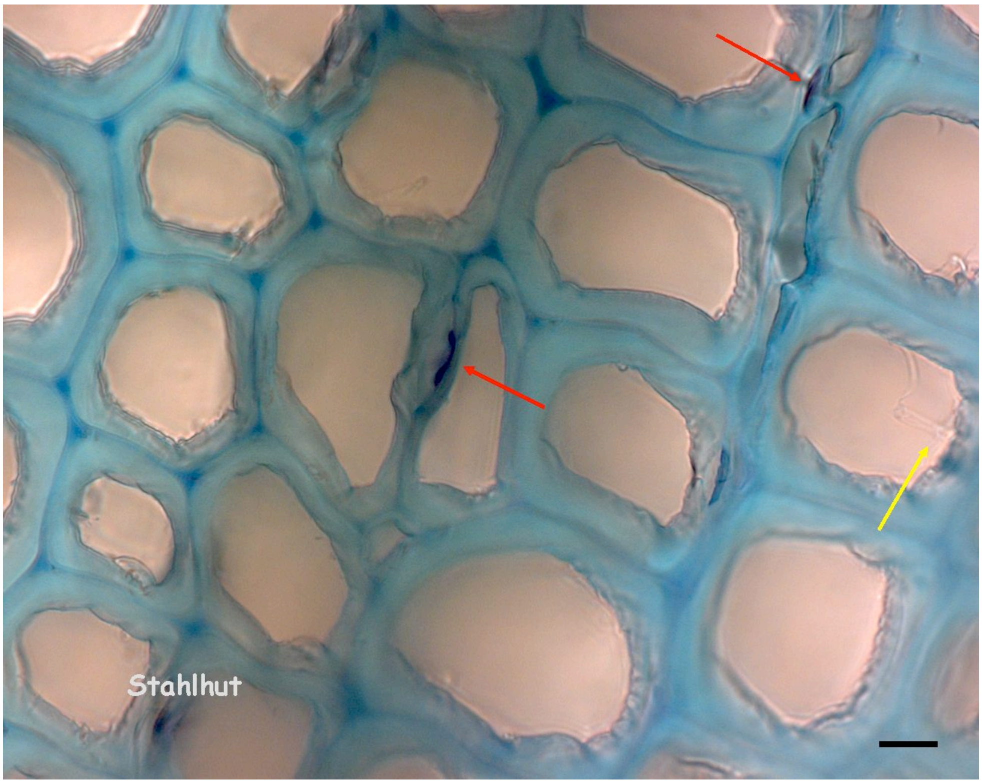


Figure 5.29 Transverse section showing toluidine blue stained and purple appearing bordered pit membranes (red arrows) in intact latewood cells and *Oligoporus placenta* hyphal early colonisation (yellow arrow). Scale bar 10µm.

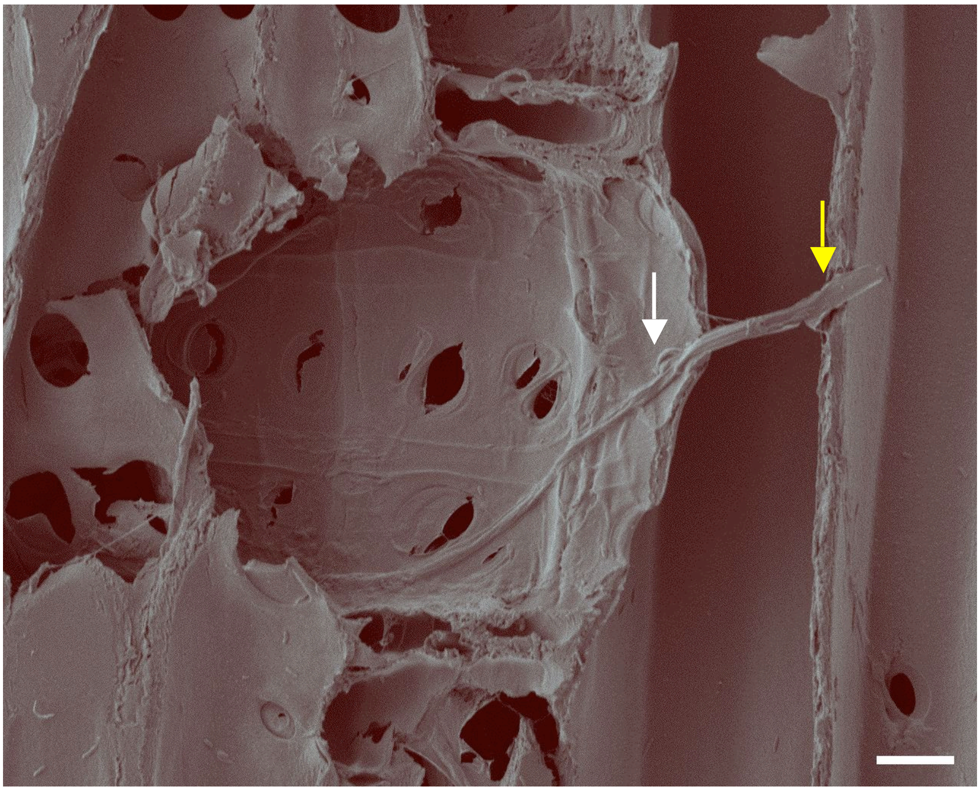


Figure 5.30 *Oligoporus placenta* advancement through degraded ray pit field in radial view. Hyphae with ordinary clamp connection (white arrow). Note: Hyphal movement into axial tracheid, possibly a cutting artefact where the hyphae settled onto the cell wall (yellow arrow). Scale bar 10µm.

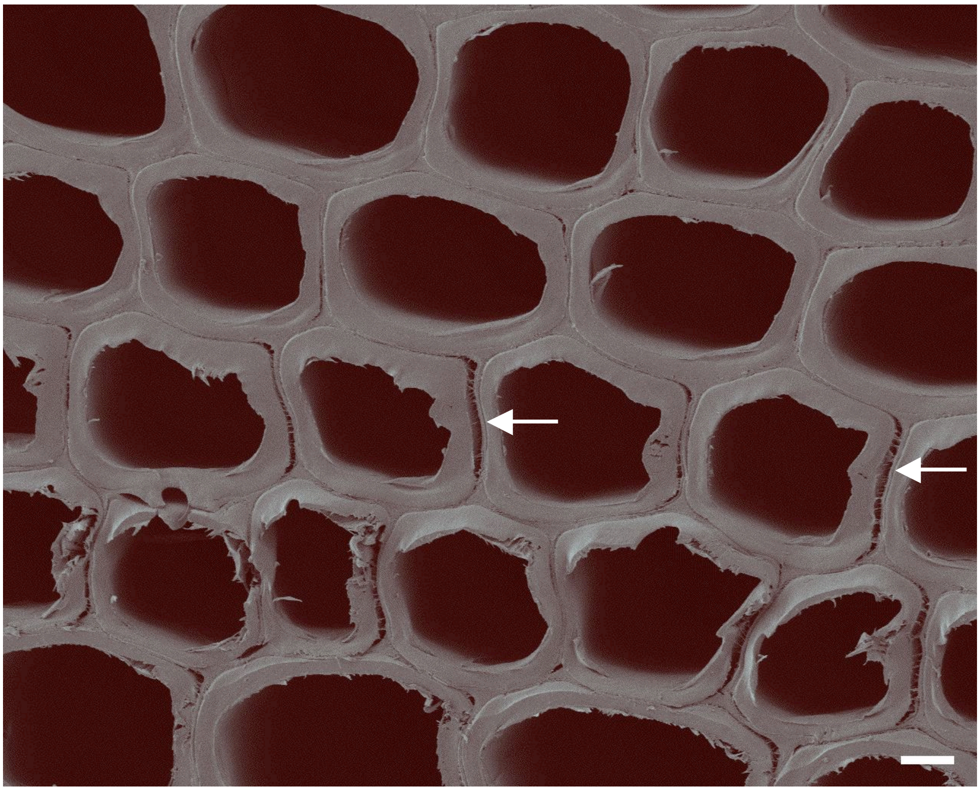


Figure 5.31 Transverse section showing early delamination of cells at the middle lamellae/S2 layer interface (white arrows) caused by *Oligoporus placenta*. Scale bar 10µm.

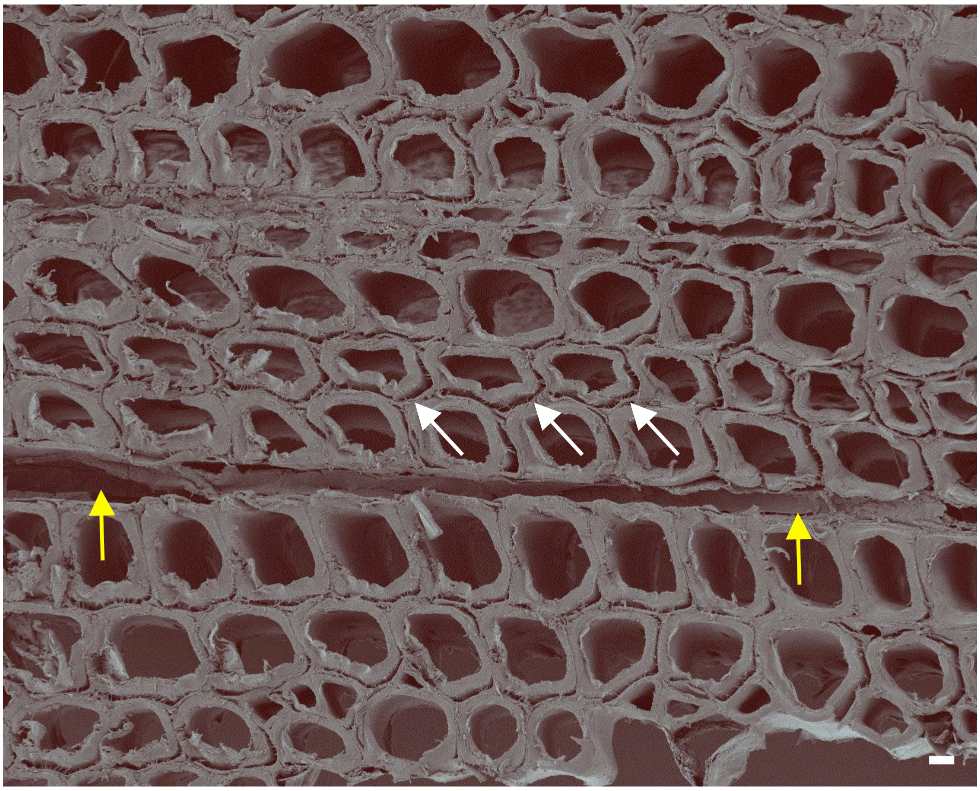


Figure 5.32 Transverse section showing advanced stage of delamination at middle lamellae/S2 layer interface with early cell deformation (white arrows) and partly degraded wood ray (yellow arrow) caused by *Oligoporus placenta*. Scale bar 10µm.

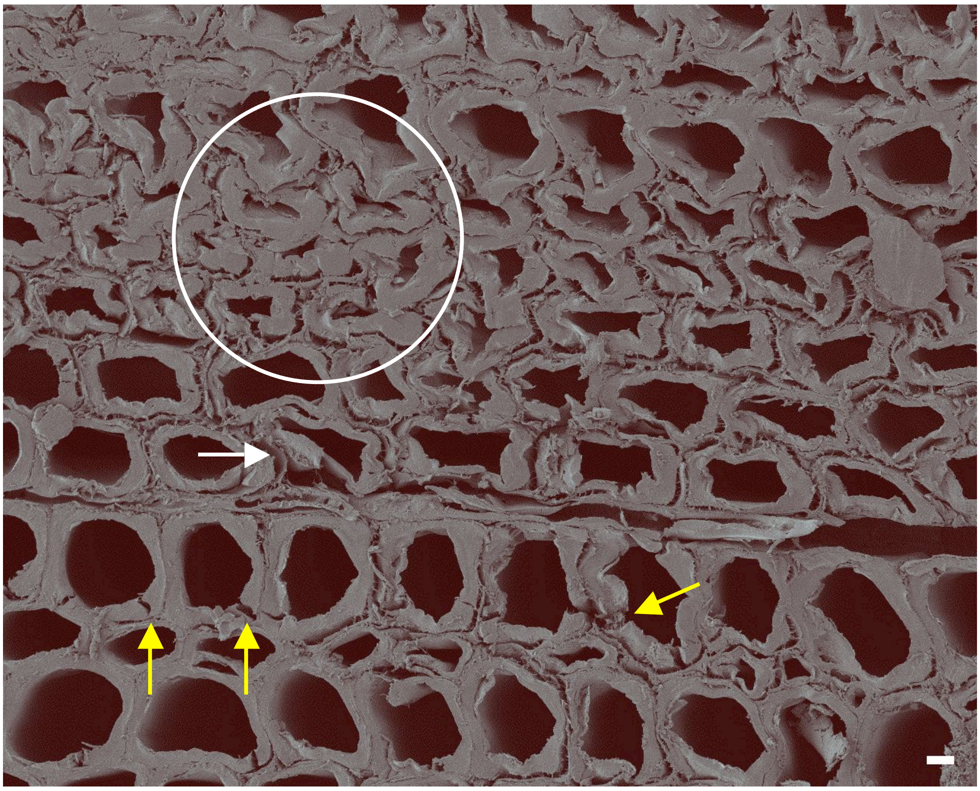


Figure 5.33 Transverse section showing advanced stage of cell deformation (white circle), cell buckling (white arrow) and cell wall breakage (yellow arrow) caused by *Oligoporus placenta*. Scale bar 10µm.

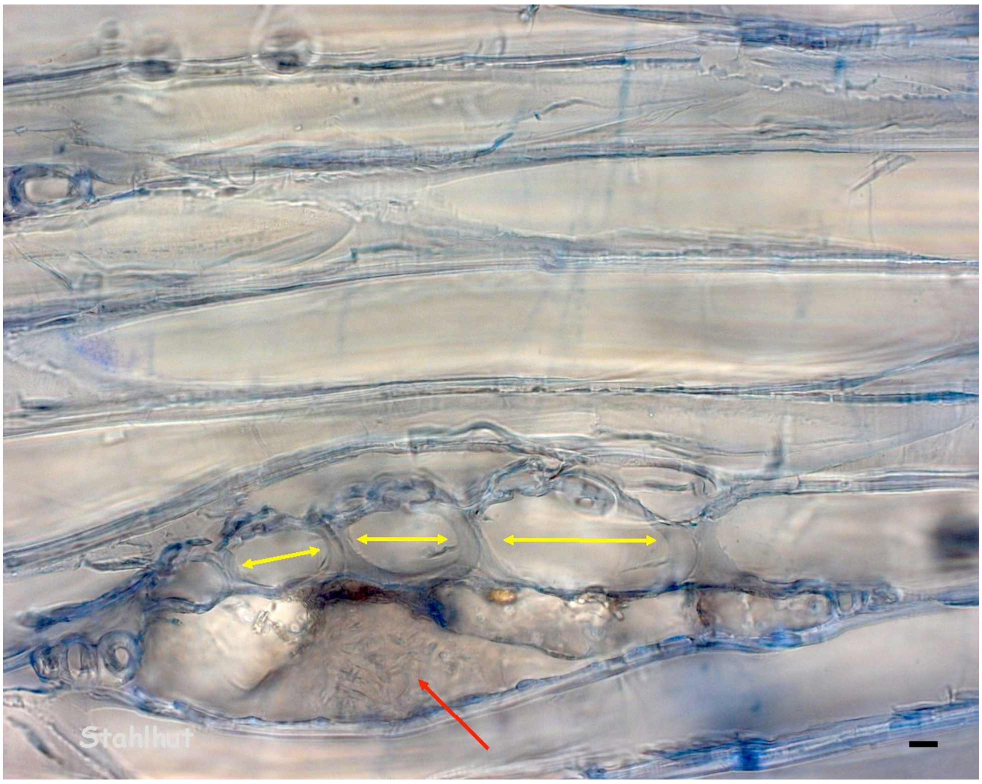


Figure 5.34 Tangential section showing undegraded ray parenchyma cells (yellow arrows) and ray colonisation by *Oligoporus placenta* (red arrow). Scale bar 10µm.

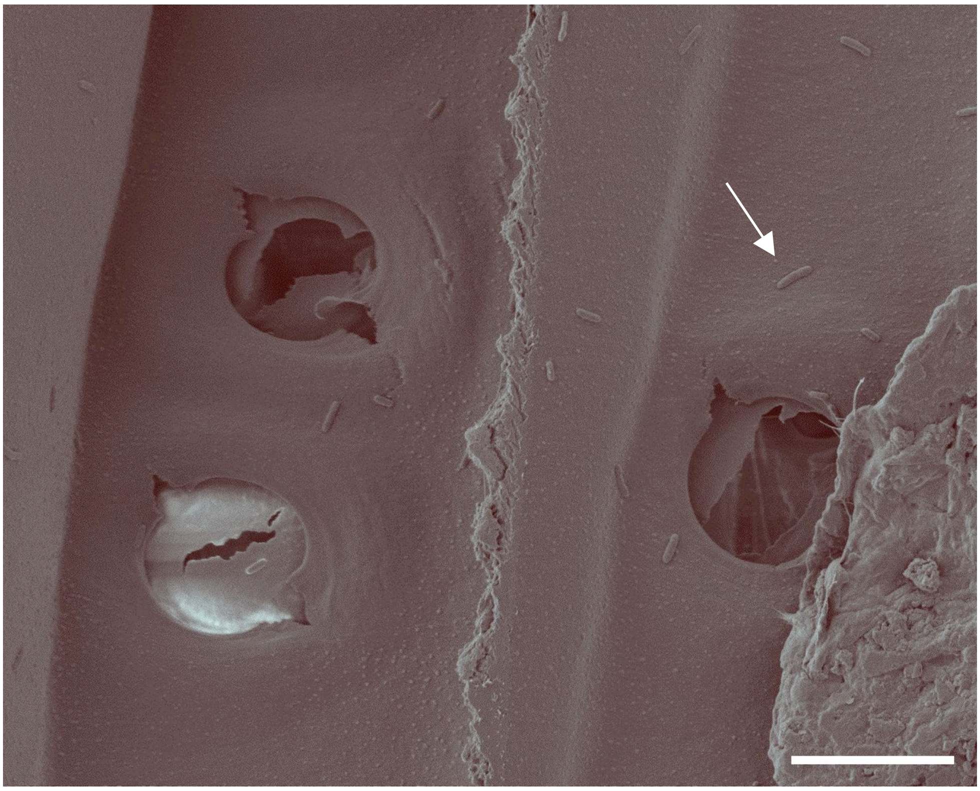


Figure 5.35 Bordered pits in radial section showing degraded (on the right), partly degraded (top left) and nearly intact (bottom left) pit membranes. Note: no *Oligoporus placenta* hyphae but bacteria present sitting on the S3 layer and pit membrane (white arrow and bottom left pit). Scale bar 10µm.

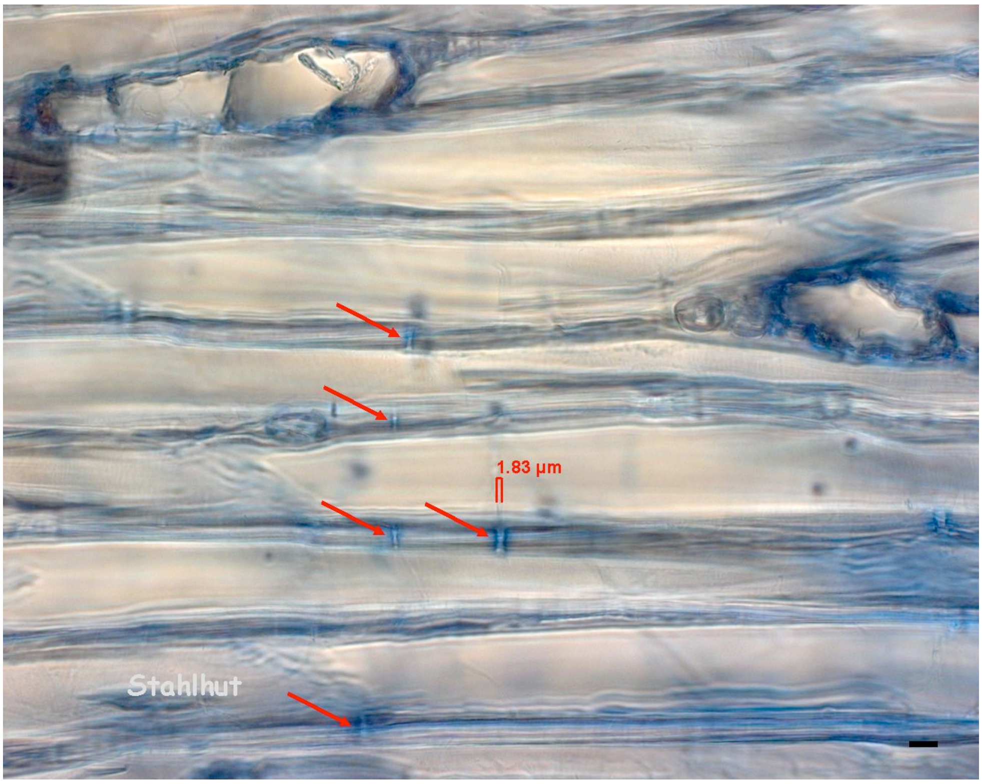


Figure 5.36 Tangential section showing bore-hole frequency (red arrows) and *Oligoporus placenta* hyphal measurement at bore-hole. Scale bar 10µm.

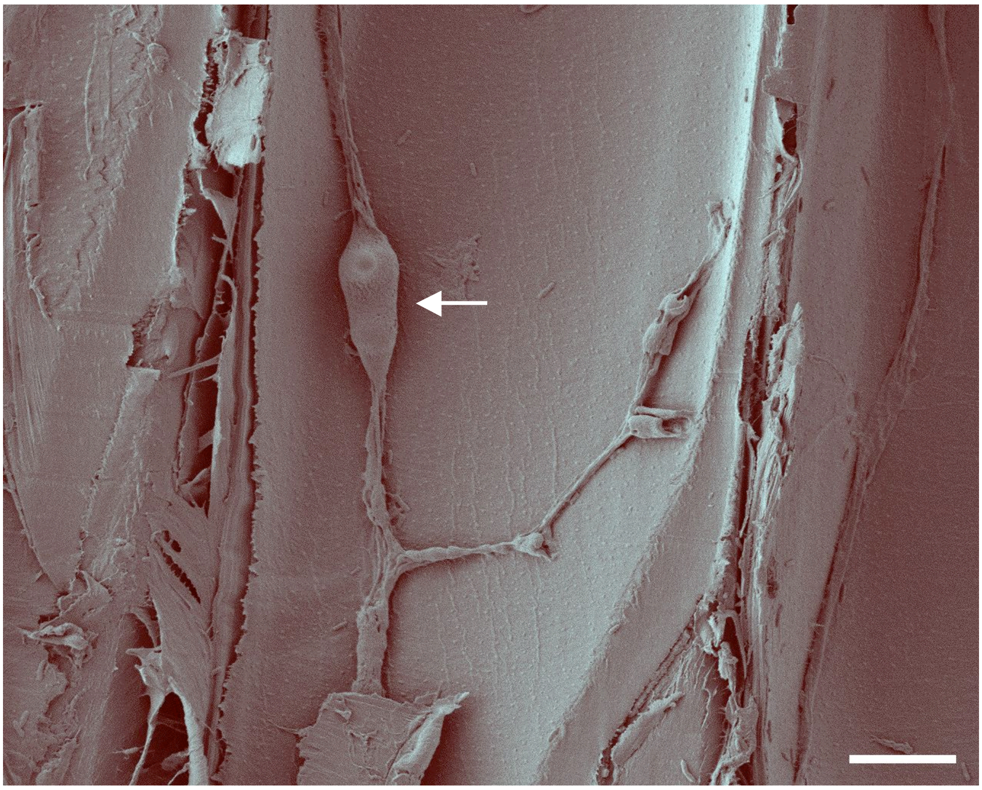


Figure 5.37 Exposed tracheid in tangential section showing chlamydospore formation in *Oligoporus placenta* (white arrow). Scale bar 10µm.

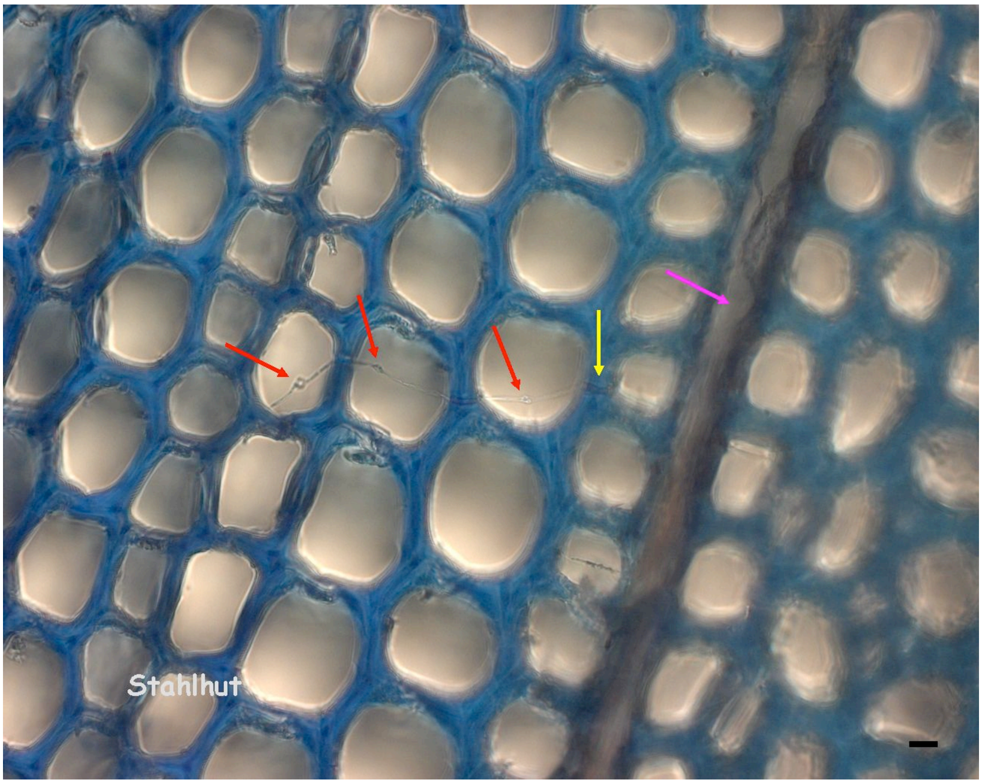


Figure 5.38 Transverse section showing *Oligoporus placenta* medallion clamps (red arrows), bore hypha (yellow arrow) and degraded ray (pink arrow) in earlywood. Scale bar 10µm.

5.3.2.4 Observations for *Gloeophyllum trabeum* colonised wood blocks

Wood blocks colonised by *G. trabeum* showed macroscopically no obvious degradative patterns such as splitting or extensive shrinkage as had occurred for the other three test fungi (Figure 5.1). This was surprising given that the mass loss data for these samples was 16% mass loss, greater than the 11% mass loss that had occurred for *S. lacrymans* which did show degradation by both microscopic observations.

The correlated and complementary data obtained from the light- and scanning electron microscopy revealed that extensive colonisation of the wood blocks had occurred, with many hyphae growing within the wood rays and spreading out into surrounding axial tracheids (Figure 5.39). Within the tracheids, hyphae branched frequently throughout the cell lumen. As was observed with the *S. lacrymans* colonised wood blocks, some ray parenchyma cells and pit fields were still detected (Figures 5.40 and 5.41). Although the middle lamellae and S3 layer were intact for most cells in early- and late- wood cells, early degradation of the S2 layer was not uncommon (Figure 5.42), leading to cell separation, distortion, buckling and breakage (Figure 5.15). Earlywood cell wall thickness was recorded as unchanged with 7.1 ± 1.6 µm.

G. trabeum, together with *O. placenta*, were the two fungi in this study to produce many bore-holes in close proximity (Figures 5.43 and 5.44) and most often were detected in tangential sections. The difference to all other brown rot decay test fungi was that hyphae of *G. trabeum* penetrating through the bore-holes were wider in diameter at the corresponding lumen entry or exit sites (Figures 5.47 and 5.48). The minimum bore-hole diameter recorded was 1µm and the widest 4.5µm, whereas the actual hyphae sizes ranged in diameter between 1µm for the thinnest and 5µm for the thickest, with many thin hyphae in most tracheids (Figure 5.47). Pit membranes, both bordered and simple, were degraded to a great extent, with many fine hyphae in close proximity (Figures 5.45 and 5.46).

Round clamydospores were found deposited in rays and tracheids (Figures 5.41, 5.43 and 5.47) with a diameter range of 4.5µm to 5.5µm, being slightly bigger than those recorded for *O. placenta*. Ordinary clamp connections were few compared to *A. sinuosa* and *O. placenta* (Figure not shown) and like *S. lacrymans* did *G. trabeum* not form any medallion clamps. Even though mass loss and decay patterns were similar to those of *S. lacrymans*, *G. trabeum* was the third fungus where slime/mucilage surrounding fine hyphae occurred (Figure 5.52, Section 5.3.3).

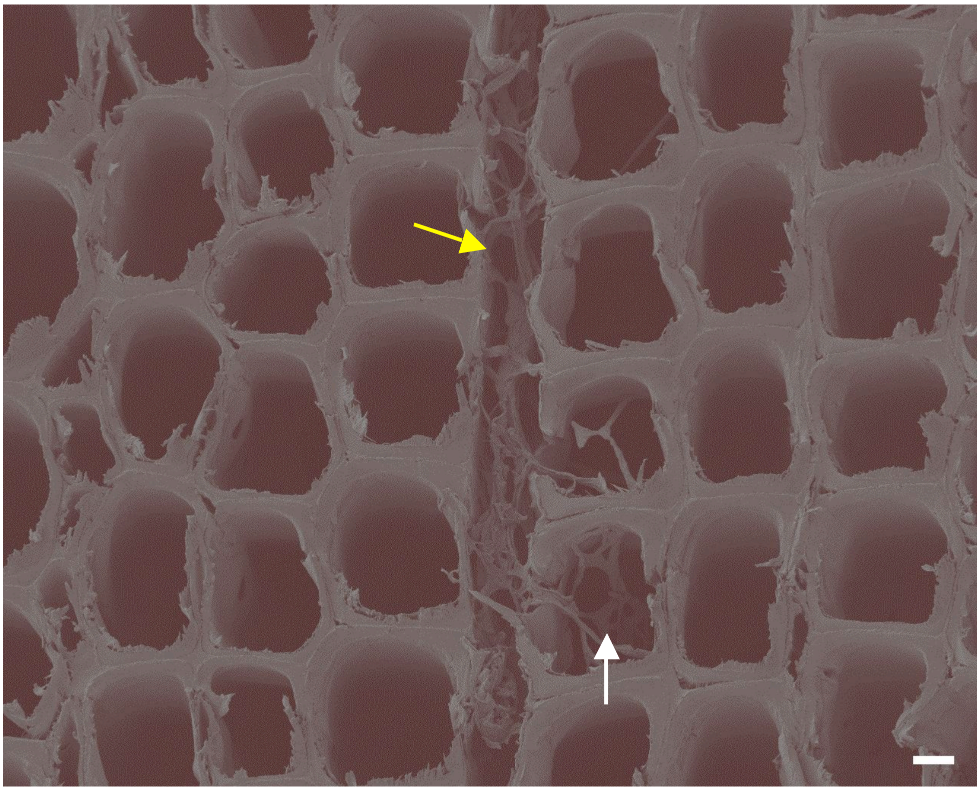


Figure 5.39 Transverse section showing extensively degraded ray parenchyma cells with large masses of *Gloeophyllum trabeum* fungal growth (yellow arrow) and advancement into surrounding tracheids (white arrow). Scale bar 10µm.

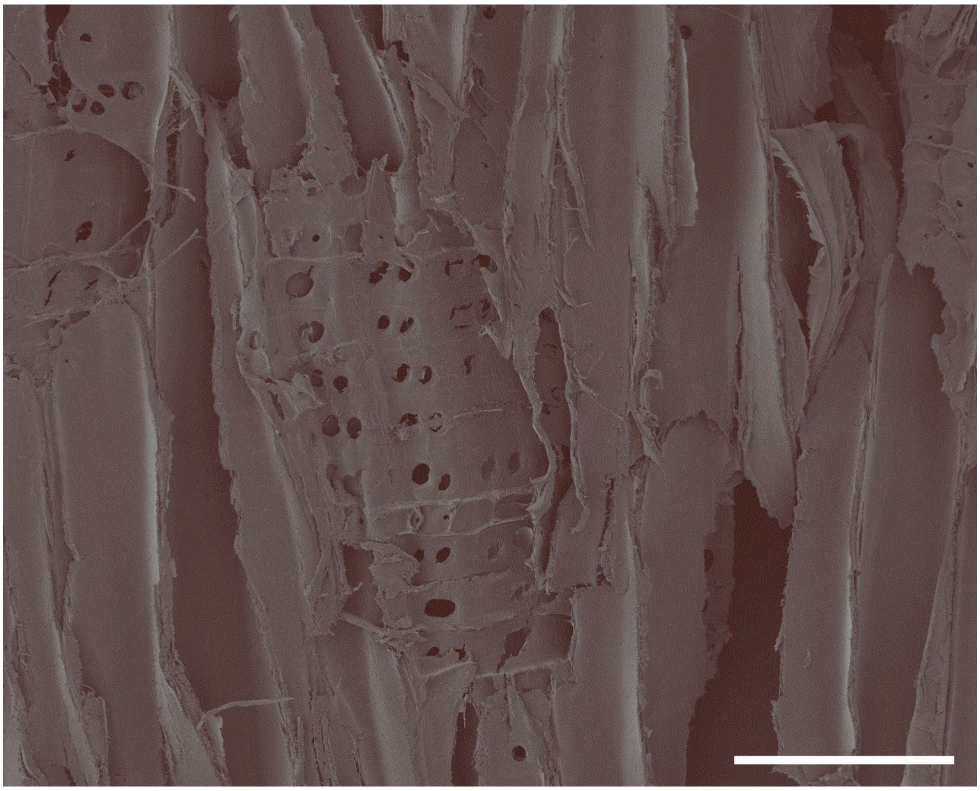


Figure 5.40 Radial section exposing *Gloeophyllum trabeum* degraded ray pit field. Scale bar 100µm.

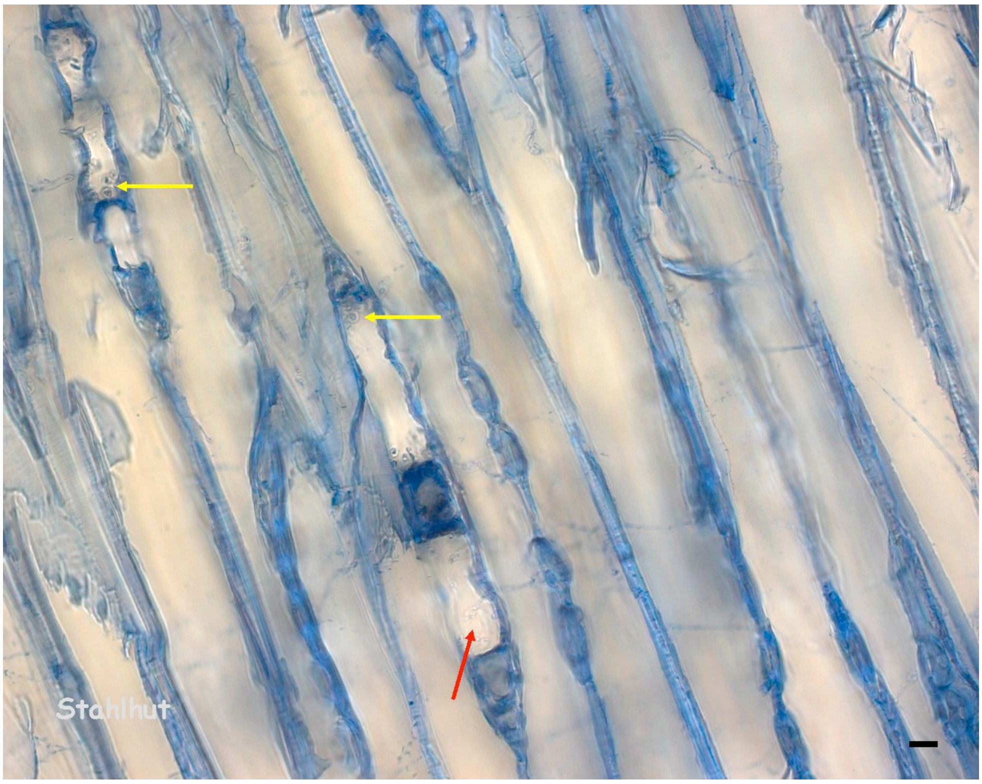


Figure 5.41 Tangential section showing partly degraded ray parenchyma cells containing chlamydospores (yellow arrows) and hyphae of *Gloeophyllum trabeum* (red arrow). Scale bar 10µm.

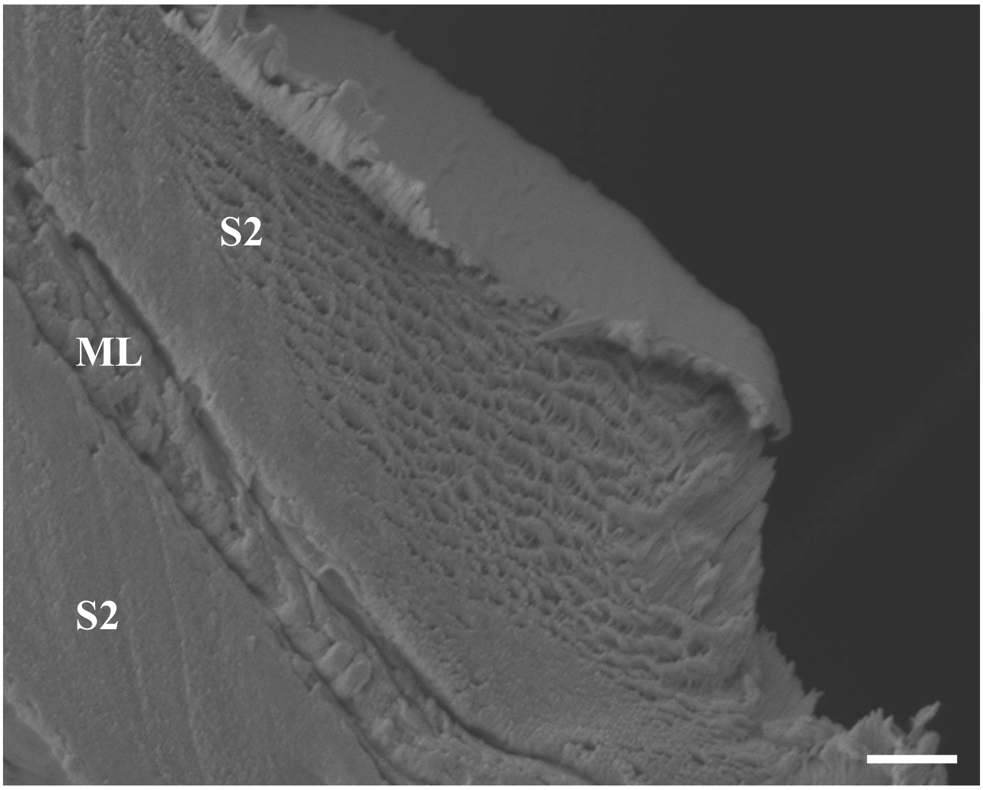


Figure 5.42 Early signs of cellulose/hemicellulose degradation by *Gloeophyllum trabeum* in the upper S2 cell wall layer (transverse section), which is distinctively porous. ML= middle lamella. Scale bar 1µm.

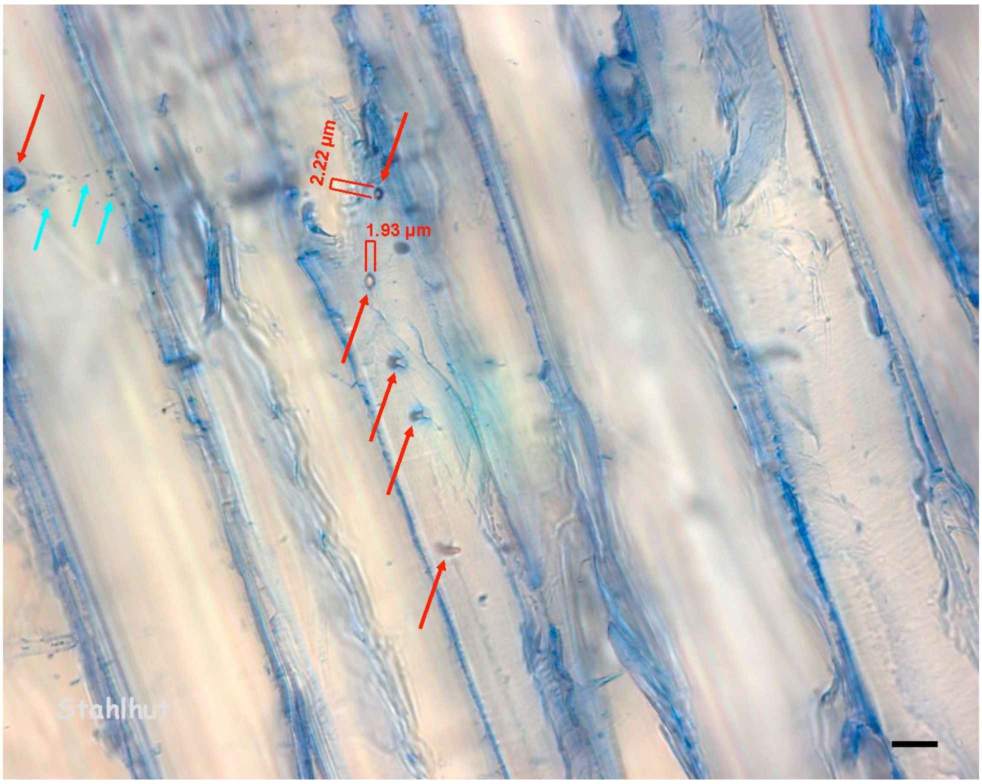


Figure 5.43 Tangential section outlining bore-hole frequency (red arrows) and measurements, chlamydospore deposition (red arrow top left corner) and slime/mucilage surrounding hyphae of *Gloeophyllum trabeum* (turquoise arrows). Scale bar 10µm.

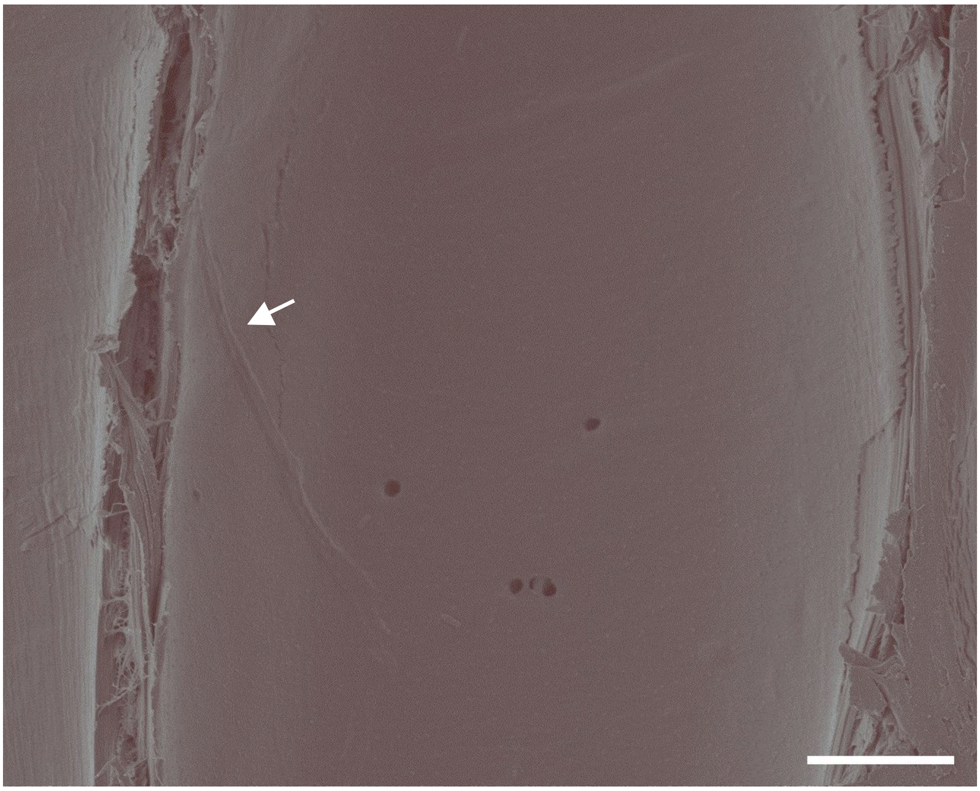


Figure 5.44 Tangential section showing fine bore-holes in tracheid and *Gloeophyllum trabeum* hypahe growing on the S3/warty layer (white arrow). Scale bar 10µm.

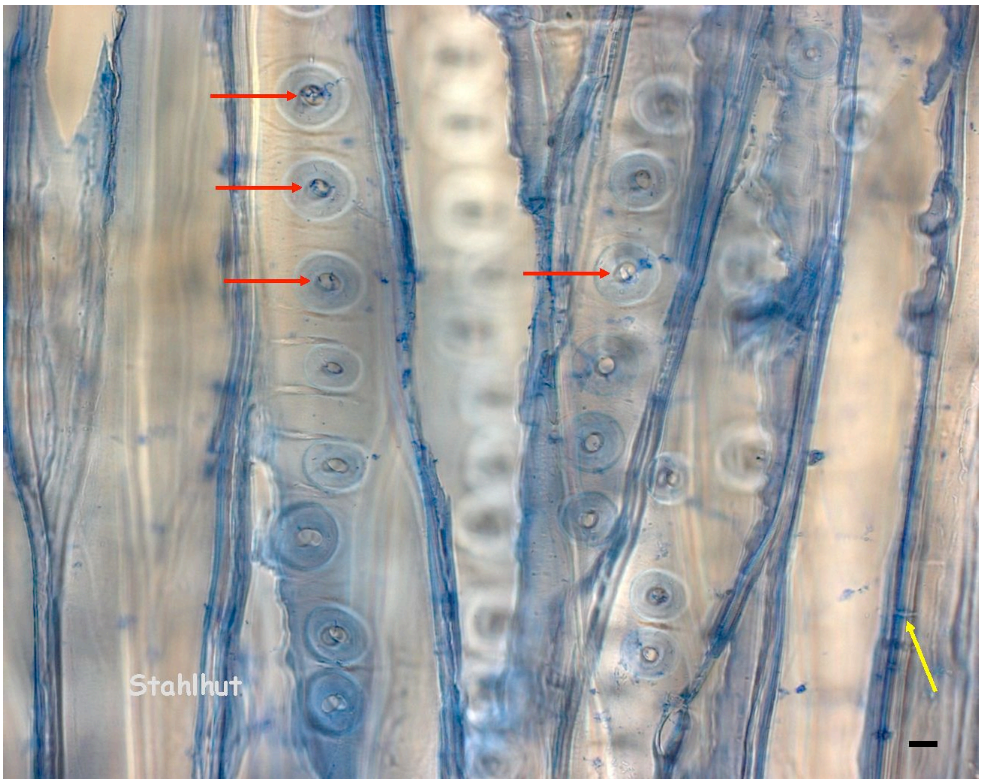


Figure 5.45 Radial section showing degradation of bordered pits by *Gloeophyllum trabeum* (red arrows) and bore-hole (yellow arrow). Scale bar 10µm.

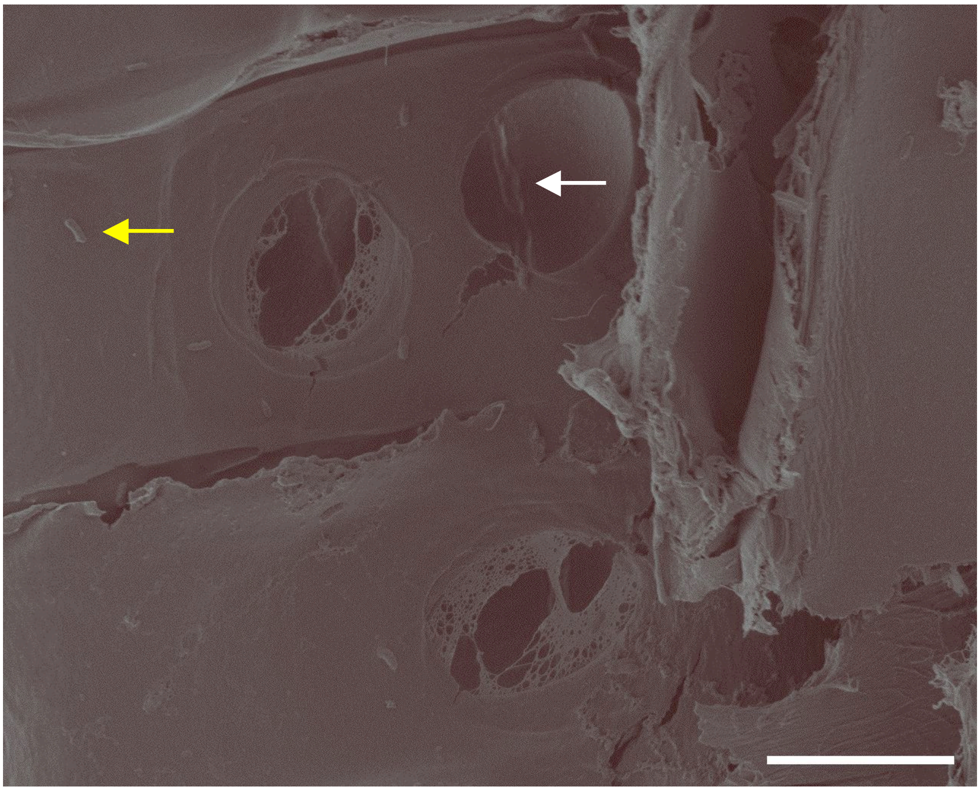


Figure 5.46 Degradation of pit membranes in radial section. Fungal hypha of *Gloeophyllum trabeum* growing in tracheid (white arrow) and bacteria (yellow arrow). Scale bar 10µm.

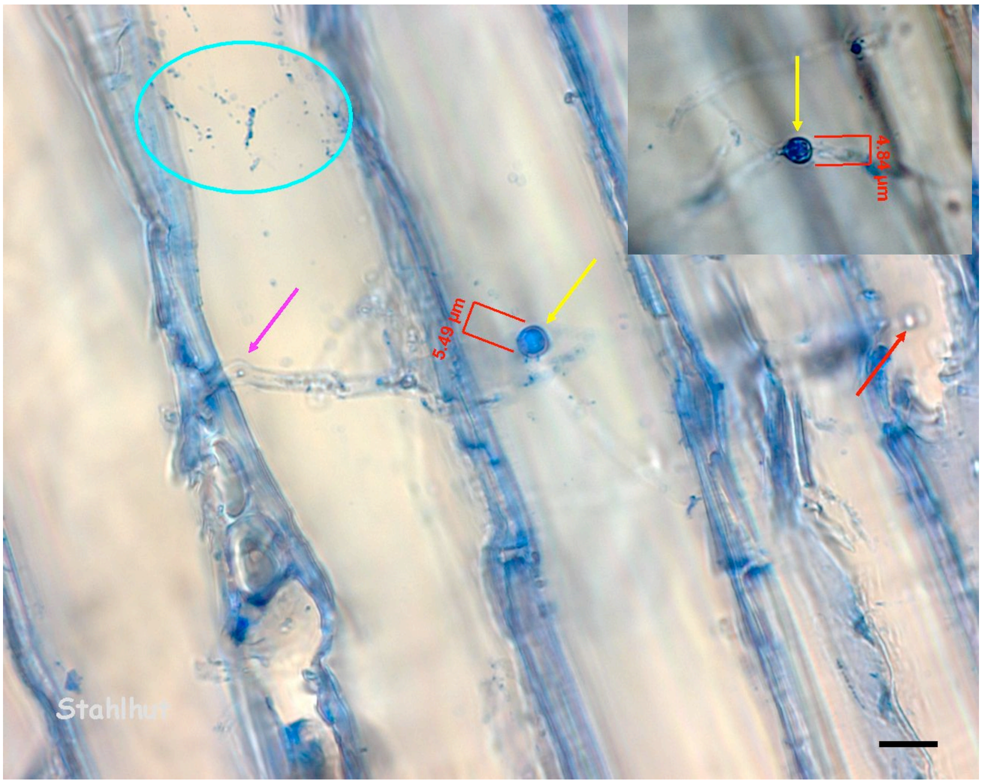


Figure 5.47 Tangential section exposing tracheids with bore-hole (red arrow), chlamydospore deposition and measurement (yellow arrow), bore hyphae (pink arrow) and slime/mucilage surrounding fine *Gloeophyllum trabeum* hyphae (turquoise oval). The inset shows chlamydospore (yellow arrow). Scale bar 10µm.

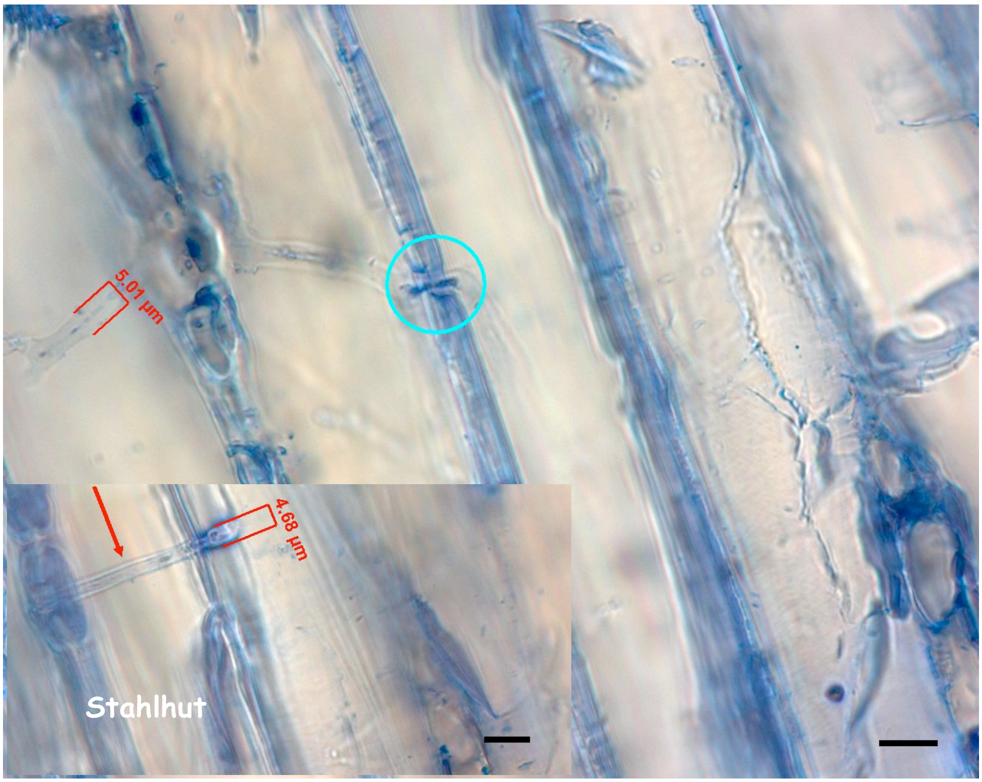


Figure 5.48 Tangential section highlighting bore hyphae 'squeezed' through the bore-hole (turquoise circle) and measurement of very wide hyphae of *Gloeophyllum trabeum*. The inset shows hyphal migration through bore-hole and bordered pit (red arrow). Scale bars 10µm.

5.3.3 Discussion

After the twelve weeks exposure of the *P. radiata* test blocks to the four test fungi, their macroscopic appearance showed all facets of known brown rot resemblance. This ranged from no obvious degradative patterns and little shrinkage for *G. sepiarium,* to deep cross cracking and splitting with a brownish powdery appearance due to extensive shrinkage for *A. sinuosa*. Further, decay in restricted pockets within the earlywood occurred in wood degraded by *S. lacrymans* and *O. placenta* combined with extensive shrinkage for the latter and early splitting for the former. Brown rot fungi do not always produce large uniform decay areas of readily detectable decay; they more often produce an internal form of decay within structural timber that depends on the fungus and wood species making it more difficult to detect (Zabel & Morrell 1992; Eaton & Hale 1993). Decay of softwood by *S. lacrymans* usually results in a dry cubical rot presenting deep cracks across and along the grain (Grosser 1985; Eaton & Hale 1993). This can explain why the here tested strain of *S. lacrymans,* which caused only minor mass loss in the range of 8-14%, had deep cracks not shaped but beginning to show around the edges of the blocks. In contrast, *G. trabeum,* with a broad mass loss range of 6-22%, showed the early signs of yellowish/brownish discolouration known to be caused by *Gloeophyllum* sp. No common decay pockets or splitting as would occur in later decay stages by *Gloeophyllum* sp. (Grosser 1985) were clearly distinguishable.

Besides radial fungal penetration in ray parenchyma cells and longitudinal colonisation of tracheids, hyphae of all four brown rot decay fungi advanced from cell to cell via pit apertures (both bordered and simple cross-field pits) and by penetrating across cell walls via bore-hole producing hyphae. The ability of the fungus *S. lacrymans* to directly locate and subsequently open and penetrate into neighbouring cells is illustrated in Figures 5.49 (penetration through ray pits) and Figure 5.50 (ray colonisation and advancement into tracheids using bordered pits). It is therefore deduced that through hydrolyses of wood pectin from the tori of the pit membranes, brown rot fungi open up the main pathway to colonise the wood (Daniel 1994; Green & Highley 1997). Since pit membranes are non-lignified they represent a more available source of carbohydrates including pectin and cellulose (Green *et al.* 1995; Schwarze 2007).

From the literature, many decay test studies showed, especially for early decay, that most brown- and white-rot fungi have the capacity to hydrolyse the pectin in pit membranes (Wilcox 1978; Green *et al.* 1995; Green & Clausen 1999; Schwarze & Landmesser 2000). In *O. placenta* test blocks in this PhD thesis research, the fungus was seen to ramify into most of the early- and some of the late- wood cells, often via the pits (Figure 5.50), but also by bore-hole penetration. Early studies of Cowling (1961) and Wilcox (1978) into incipient stages of decay on wood strength showed that hyphae of *Oligoporus* sp. rapidly colonised an entire test block via simple and bordered pits, before a measurable mass loss of 5% had occurred. However, this should not generally be seen as the norm, as especially latewood cells are colonised later in the decay process as here shown in Figure 5.26, where some intact bordered pit membranes were detected even though the overall mass loss for the block was recorded with 33%. Similar observations by Eriksson *et al.* (1990) detecting many unimpaired bordered pits in later decay stages supports this view and outlines that fungi preliminary use the most suitable, with little hindrances, way to ramify into the wood. Hence latewood cells consist of fewer bordered pits, are thicker walled and enclose a narrow cell lumen, they are colonised at a later stage.

That wood cells which are denser have a lesser risk to be degraded early on in the brown rot decay process was shown in a study by Schwarze & Spycher (2005). *Picea abies* test blocks were thermo-hygro-mechanically (THM)-densified, with the result that the engineered wood product significantly restricted *C. puteana*, *G. trabeum* and *O. placenta* brown rot colonisation and degradation. Following microscopical examinations revealed that the differences between THM-densified wood and controls could be partly attributed to the occlusion of tracheid lumina restricting brown rot fungal growth (Schwarze & Spycher 2005). On the contrary, it was shown that soft rot fungi could still decay THM-densified wood since their hyphae grow and colonise wood often within secondary walls.

That brown rot hyphae predominantly grow within the cell lumen or attached to the lumina layer, instead of directly eroding the cell walls like soft rot fungi, has often been discussed in the past (Rayner & Boddy 1988; Eriksson *et al.* 1990; Zabel & Morell 1992; Eaton & Hall 1993; Schmidt 2006), including enzymatic pit membrane degradation and penetration by hyphae to rapidly ramify into the wood.

The results of this PhD thesis research demonstrated, surprisingly, that fungal hyphae 'locate' pits and seemingly grow straight towards them, avoiding the long way round following the luminal layer (as observed in Figure 5.50) and this to our knowledge has not been previously published. When looking at the hyphae growing within the lumen, as shown in Figure 5.49, there are no other induced movements such as (T-) branches, instead it becomes obvious that in this case pit openings are directly targeted by the 'mother' hyphae. Hence, the fungal hyphae do not necessarily need close proximity to the cell walls to cause decay (Daniel 2003) and assuming hydrolyses of the non-lignified pit membrane opens up new territory, it can be hypothesised that through this precursor action the triggered response is possibly new apical growth targeted towards the degraded pit structure.

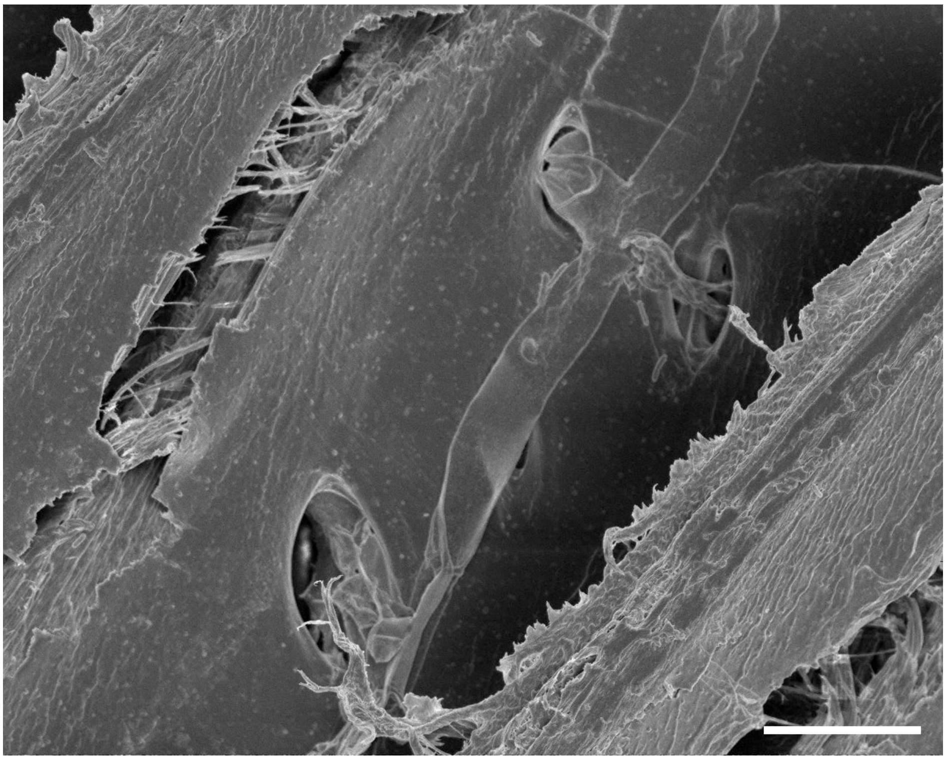


Figure 5.49 Radial section showing hyphal growth of *Serpula lacrymans* in ray parenchyma with movement through simple cross-field pits. Scale bar 10μm.

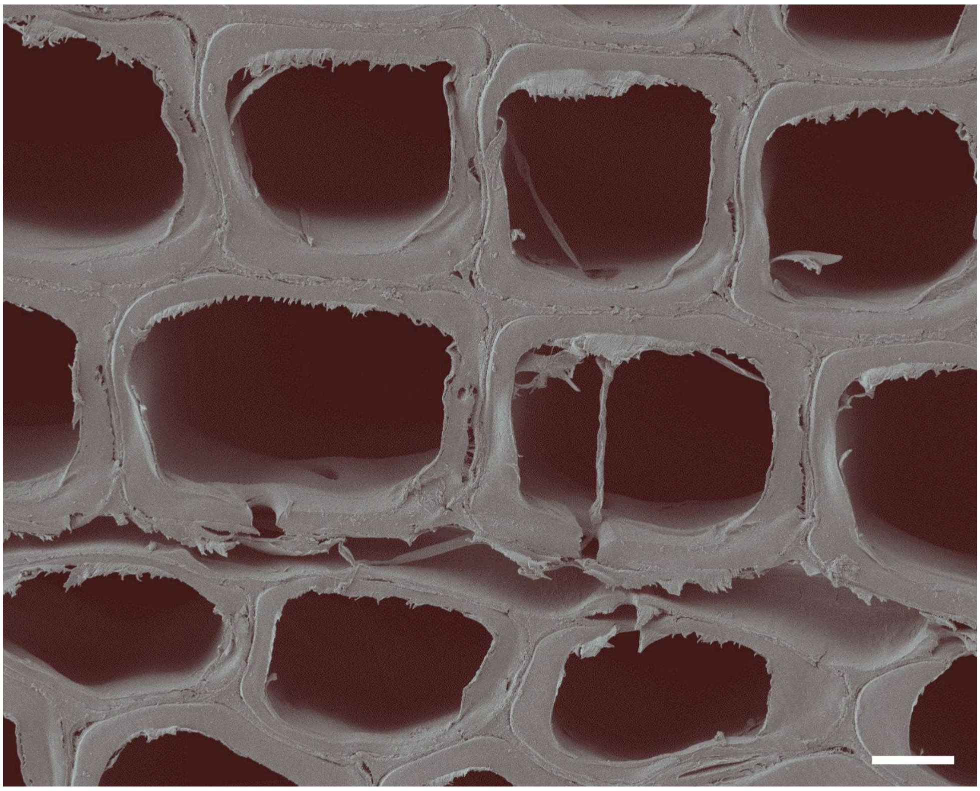


Figure 5.50 Transverse section showing hyphae of *Oligoporus placenta* growing out of the ray parenchyma cells into tracheid through pit openings. Scale bar 10μm.

Brown rot fungi possess the ability to utilize hemicellulose and cellulose early on in the decay process without first removing the protective and encrusting lignin (Green & Highley 1997). Even at low mass losses, as had occurred for *G. trabeum* and *S. lacrymans*, a rapid decrease in the degree of polymerisation is caused (Green & Highley 1997; Daniel 2003). As a result of fungal attack and its subsequent progress, several features or early signs can be observed microscopically throughout the wood cells interconnection through their respective change in state. Features such as early cell wall delamination at the S2/S1/primary wall/middle lamella interface (Figure 5.31) occur, possibly due to lower lignin content of the S1 compared to the S2 in *P. radiata* (Donaldsen 2001). This is followed by trans-wall fractures such as cell distortion, buckling or breakage at later stages (Eriksson *et al.* 1990; Wilcox 1993a) possibly due to both, shrinkage when dry, and the cell walls inability to withstand mechanical cutting forces as observed in Figure 5.33. Additional cell wall degradation is accompanied by occurrence of bore-holes, observed in Figure 5.44 and the loss of cell wall birefringence due to the cell walls loss of crystalline compounds (e.g. cellulose) (Eriksson *et al.* 1990; Wilcox 1993a; Anagnost 1998; Schwarze 2007).

Many authors have referred to the use of polarised filters to determine loss in birefringence as a valuable tool in the diagnoses of brown rot attack (Wilcox 1993a; Anagnost 1998; Daniel 2003), with Schwarze (2007) calling this method superior to any other diagnostic approach. However, care should be taken to not rely on the degree of birefringence, in part, because orientation of the sections (tangential/radial/transverse) and their thickness can impair reliability. Wilcox (1993a) pointed out that loss in birefringence could be observed for *O. placenta* at 3% mass loss, but that for *G. trabeum* this was not detected until more advanced stages of decay, suggesting a different mode of action for these two fungi. Further he emphasised that loss in birefringence is best observed in very thin transverse sections, which are difficult to obtain without prior embedding of the wood sample. The fairly thick (90µm) and stained wood sections used in this correlative LM and FE-SEM study, was one reason why birefringence was not included as a feature in the summary Table 5.1. This problem is illustrated in Figure 5.51, which shows no distinct differences of birefringence across the cell walls using crossed polarising filters. However, the absence of any change in birefringence does not imply that it wasn't present; it may simply not occur in the small sample of wood chosen for examination (Anagnost 1998).

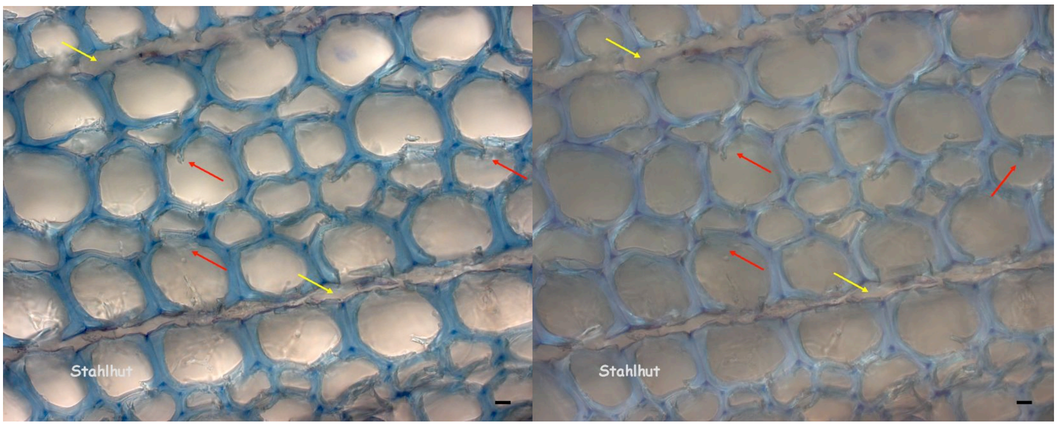


Figure 5.51 Transverse section photorgraphed under phase contrast (left) and polarisation (right). Cell wall fractures (red arrows) and partly degraded rays (yellow arrows) after 12 weeks of colonisation by *Serpula lacrymans*. Scale bar 10µm.

Microstructural and ultrastructural studies on wood degraded by brown rot fungi have shown that a close proximity between hyphae and the wood cell wall is not necessary for cellulose and hemicellulose depolymerisation (Daniel 2003). Huckfeldt (2003) called this classic brown rot decay mechanism, which does occur in all brown rot fungi, as depolymerisation from a distance. A second mechanism that does include complete cell wall degradation including the lignin rich middle lamella and seen as bore-holes, has been referred to as proximity degradation (Huckfeldt 2003). The difference in the latter mechanism is that the fungus produces very fine bore hyphae (Wilcox 1993a) directly penetrating the cell wall. These bore hyphae (and hyphae within the lumen), when observed by electron microscopy, are sometimes surrounded by a thick slime layer (hyphal sheat) (Huckfeldt 2003) thought to play a major role in enzyme and low-molecular mass transfer to the wood cell wall (Schmidt 2006; a list of possible functions is provided in Section 1.3.1.1).

All four brown rot fungi in this study produced in colonised *P. radiata* wood specimens bore-holes ranging in size for very small ones of 1µm to 3.5-5µm for larger ones. Additionally, for all except *S. lacrymans*, fine hyphae were frequently surrounded by slime layer (Figures 5.52 and 5.53). These slime layer are also known as extracellular mucilaginous material (ECMM) which is secreted at the tips of fine hyphae, indicating active growth (Evans *et al.* 1981) and was suggested to facilitate extended protection to the young hyphae (Vesentini *et al.* 2006). The production of ECMM was shown to increase for *T. versicolor* and *G. trabeum* after environmental stress (growth conditions), including pH, temperature and low nutrient availability (Vesentini *et al.* 2005). Further, Vesentini *et al.* (2006) showed that particularly the numbers of actively growing hyphal tips is likely to have a significant bearing on ECMM production. Therefore, absence of a slime layer in *S. lacrymans* degraded wood samples might be attributed to the limited amount of sections examined (n = 12), coupled with the limited number of hyphae/tips present within the tracheids (≤1) and near optimum growth conditions.

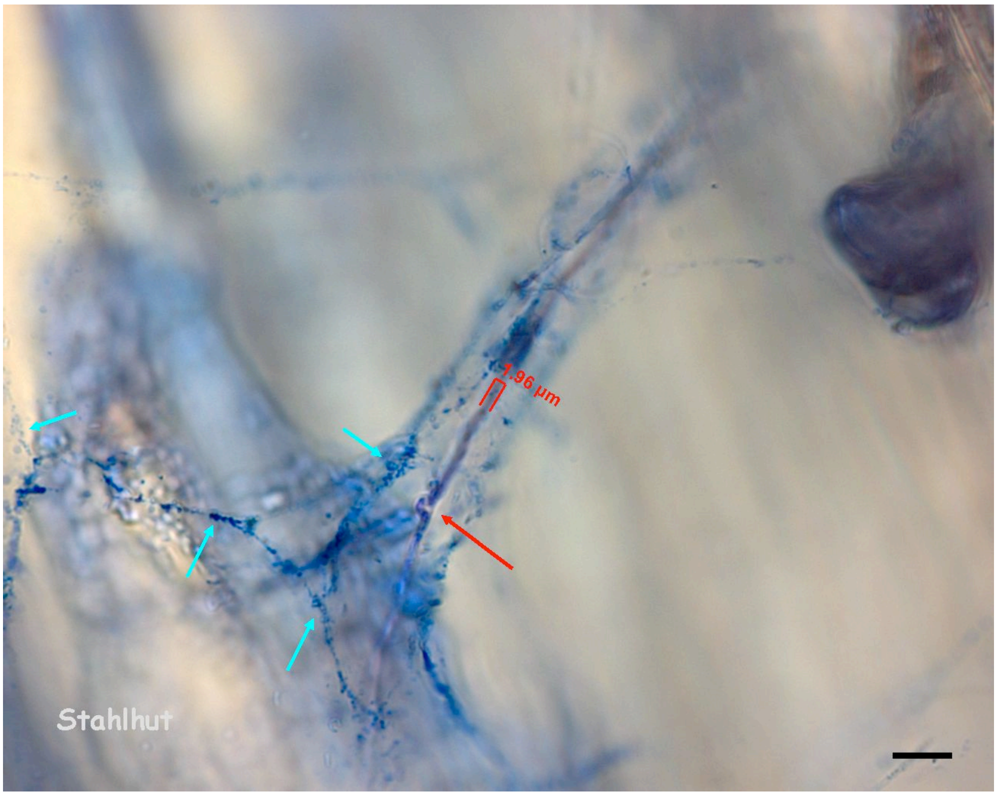


Figure 5.52 Tangential section showing slime layer surrounding thin *Gloeophyllum trabeum* hyphae (turquoise arrows) and wider hyphae with clamp connection (red arrow). Scale bar 10µm.

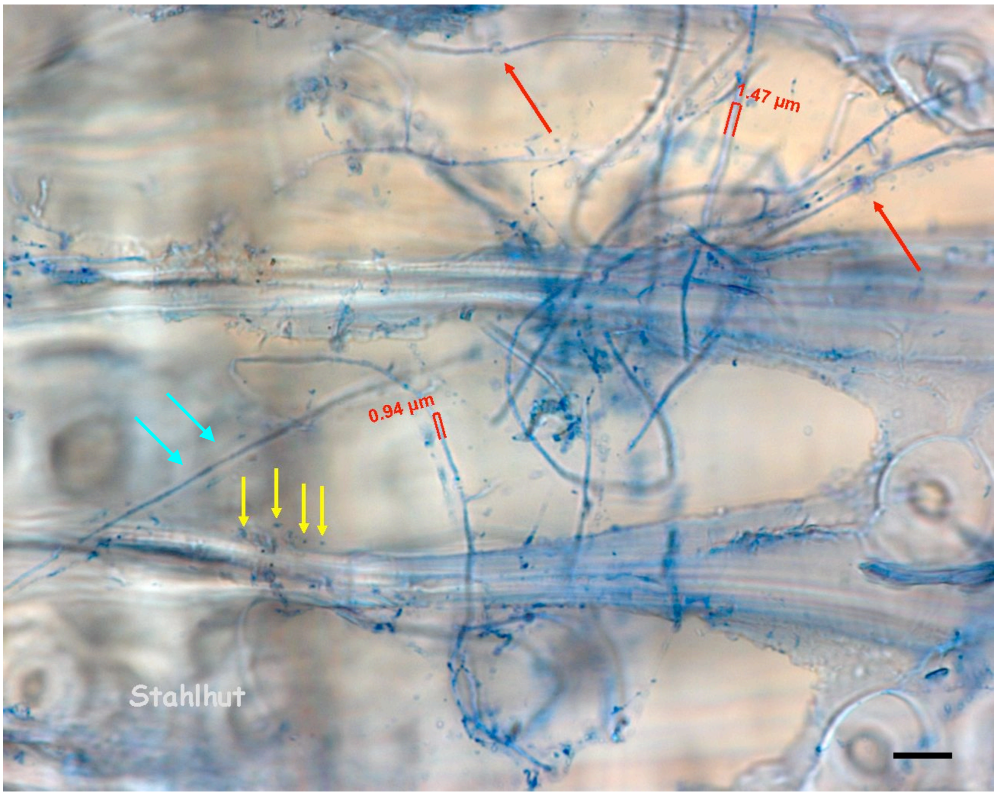


Figure 5.53 Slime layer surrounding thin *Antrodia sinuosa* hyphae (turquoise arrows), ECMM (yellow arrows), medallion clamps (red arrows) and measurements in radial section. Scale bar 10µm.

Though all four test fungi produced bore-holes, they were random and infrequent. For the fungal species tested in this PhD thesis research literature studies described them as capable to fully penetrate the cell wall (e.g. Wilcox 1993a,b; Worrall *et al.* 1997; Anagnost 1998; Huckfeldt 2003). Wilcox (1993a) reported, for example, bore-holes produced by *O. placenta* and *G. trabeum* to be apparent in the earliest stages of decay, represented by 3% mass loss. He concluded that even though the presence of bore-holes does not indicate the stage of decay, their respective size can. Further Wilcox outlined that the presence in combination of only small diameter hyphae and small bore-holes is indicative for incipient decay (Wilcox 1993a).

This study showed that for well established to advanced decay (> 10% mass loss), diameter of hyphae and bore-holes was highly variable. A second common observation is with respect to the bore-hole and corresponding hyphal size. Typically, the diameter of the bore-hole increases with progressing brown rot decay and the hyphae passing through it being smaller (Figure 5.36)(Zabel & Morrell 1992), whereas at earlier stages the hyphae might be considerably larger than the bore-hole (Figure 5.48) (Wilcox 1993a; Anagnost 1998).

Intact parenchyma cells were observed with this PhD study even though ray parenchyma cells were largely missing compared to controls, which especially accounted for *S. lacrymans* degraded wood samples at low mass losses. A common perception in softwood brown rot decay is the early colonisation and degradation of ray parenchyma cells (Figure 5.39) (Wilcox 1993a; Worrall *et al.* 1997; Anagnost 1998), with others reporting some intact parenchyma cells even at higher mass losses (Eriksson *et al.* 1990; Schwarze 1995). Schwarze (2007) hypothesised that parenchyma cells in general are resistant to brown rot fungi and that higher mass losses are directly linked to the parenchyma content in the wood species. *Picea abies* was associated with the highest mass losses and lowest parenchyma content of 5-10%, whereas hardwoods such as *Quercus* sp. and *Robinia* sp. with high parenchyma content of 35-40% showed low mass losses (Schwarze 2007). To test this theory Schwarze (2007) used the brown rot fungi *O. placenta* and *G. trabeum* and white rot fungus *T. versicolor* on *Ochroma pyramidale* (balsa), which consists of 92% parenchyma cells (Wagenführ 1999) and *P. sylvestris* as control. It was found that although blocks were highly colonised by the brown rot fungi, mass loss was negligible in balsa wood whereas high in *P. sylvestris*. Since white rot fungi preferentially degrade parenchyma cells (Schwarze & Fink 1998), mass loss caused by *T. versicolor* was higher in balsa wood than *P. sylvestris* (Schwarze 2007). These observations make it therefore crucial to compare possible ray parenchyma degradation by brown rot fungi with the control wood blocks, as especially the method of pre-conditioning of the blocks might had an impact on the parenchyma cells too. High-temperature drying with pre-steaming for example leads to epithelial and ray parenchyma cells damage (Matsumura *et al.* 1999), which could entice the observer to the conclusion of fungal degradation. Hence, the *P. radiata* blocks in this PhD thesis research were air dried; parenchyma cells missing more frequently at later stages of decay were deduced to be degraded by the test fungi.

After 12 weeks incubation, preferential decomposition of the cellulose and hemicellulose rich S2 layer of fibre tracheids were detected in all samples, with the latewood tracheids being less colonised and decayed. It is generally accepted that the compound-middle lamella and S3 layer in conifers are more resistant to both brown and soft rots whereas the S2 layer is most susceptible (Zabel & Morrell 1992). Observations proved that the middle lamella region and S3 layer were in most cells unchanged (Figure 5.7) and that even at higher mass losses in *A. sinuosa* and *O. placenta*, latewood cells appeared only a little distorted. This can possibly be related to the higher degree of cell wall lignification, which hampers diffusion of celluloytic enzymes into the cell wall, thus resembling a greater resistance towards decomposition (Schwarze *et al.* 2003).

To estimate how advanced the decomposition of the wood blocks were, measurements of the combined cell wall thickness of two adjoining cells indicated that only in *A. sinuosa* degraded blocks had minute thinning occurred compared to the controls. Although the S2 layer was highly degraded, complete cell disintegration was not apparent as the lignin initially maintained the cell shape and dimension (Zabel & Morrell 1992). Since a change in cell wall thickness does not occur until the late stages of brown rot decay (Zabel & Morrell 1992) where only a lignin skeleton remains (Eaton & Hale 1993; Green & Highley 1997), the nearly unchanged wall thicknesses of all wood/test fungi combinations, combined with the bore-hole and fine hyphae observations, further support the moderate to advanced decay stage finding.

When collecting evidence for the decay fungi advancement in *P. radiata,* it was found that all four brown rot basidiomycetes produced chlamydospores, or arthrospores within the tracheids or wood rays. Chlamydospores are unicellular, thick-walled, hyaline or brown spores that are asexually, endogenously and most often singly produced within the fungal hyphae (Rypáček 1966; Kirk *et al.* 2001). Chlamydospores first stay enclosed within the producing hyphae until it degenerates (Rypáček 1966). Two FE-SEM pictures clearly showed a collapsing *O. placenta* hypahe releasing the chlamydospore, and a fully released spore, as shown in Figures 5.37 and Figure 5.4, respectively. Figure 5.5, contrarily, showed *O. placenta* chlamydospores hyaline and thick-walled, as has been found similar in spores produced by *A. sinuosa* (Figure 5.24) and *G. trabeum* (Figure 5.47).

Since the term chlamydospore is sometimes treated unequally (Huckfeldt & Schmidt 2006a), it might be seen merely as an arthrospore (also termed oidium) of large size. The actual difference between the two is in arthrospores being thin-walled, cylindrical and are often produced in so-called ‘oidia-chains’ by simple division of the vegetative hyphae (Rypáček 1966). Nonetheless, ‘oidia-chains’ have been described in the past as constituting the simplest form of chlamydospores (Brefeld 1889), suggesting that both represent modifications of the same form (Brefeld 1889). *S. lacrymans* in this PhD thesis research produced spores that are more likely to be classified as arthrospores since their thin-walled, cylindrical and chain-like character, found deposited within *P. radiata* tracheids (Figures 5.20 and 5.21). Similar chain-like occurrence was found in *O. placenta* (Figure 5.5), but with the difference of producing thick-walled spores, the reason why they were classified as chlamydospores.

Chlamydospores like arthrospores, as described in Section 1.4.5, were found to be more resistant to heat, dryness and wood preservatives (Schmidt 2006) and are thought to be produced in an effort to secure the fungal survival during changing environments (Snell & Dick 1957; Huckfeldt 2003; Schwarze 2007). Hence these spores are often called resting spores and can be formed early on during fungal colony establishment within or on a given substrate. Marryat (1908) was one of the first who examined the basidiomycete *Pleurotus subpalmatus* growing in hanging water drop or gelatine cultures, producing interstitial spores or chlamydospores within the main hypahe after six days of inoculation. Given that the four brown rot fungi in this decay test had colonised the wood for 12 weeks, it is not surprising that resting spores had been deposited within the wood matrix.

In respect to building decay fungi and their various types of spores produced (basidiospores, chlamydo-/arthro-spores or conidia), the literature doesn't overly support their importance, here in particular to chlamydo-/arthrospores, and their possible occurrence within decayed wood. Anagnost (1998), who extensively studied basidiomycetes on softwoods inclusive of the here-examined test fungi *G. trabeum* and *O. placenta*, did not mention chlamydospore production and left this feature out of her 'key to identification of wood decays based on light microscopic features'. Another study using light microscopy for comparative morpohology of early stages of brown rot decay including the same test fungi *G. trabeum* and *O. placenta,* (Wilcox 1993) detected the same degradative features such as clamp connections or cell delamination as frequently present, but did not record any chlamydospore production of the hyphae in wood. Since we know that chlamydo-/arthrospores are more heat and dryness resistant, for example *O. placenta* can withstand heat of >80°C for 4 hours in slowly dried timber (Huckfeldt & Schmidt 2006a), this fungal characteristic feature is important to record, especially if a heat treatment as building remediation is proposed, a practice often carried out in Denmark (Schmidt 2006). A newer identification key for European strand-forming house-rot fungi (Huckfeldt & Schmidt 2006b) has been found to be the first referring to the presence of arthrospores in some keyed species, for example rarely occurring in *G. sepiarium*, however for the four tested fungi of this study no chlamydo-/arthrospores were described.

According to some references, mainly in pure culture studies, chlamydospores were recorded for *G. trabeum* (Overholts 1967; Stalpers 1978; Huckfeldt 2003), *O. placenta* (Brefeld 1889; Stalpers 1978; Micales & Highley 1989; Schwarze 2007) and *A. sinuosa* (Stalpers 1978; Schwarze 2007 for *Antrodia carbonica*).

Arthrospores have been previously observed for *S. lacrymans* and *G. trabeum* degraded wood samples (Schmidt & Moreth-Kebernik 1990; Huckfeldt 2003), with the former not known to produce chlamydospores (Stalpers 1978).

Two of the four fungi tested showed medallion clamp formations, a very distinctive feature separated form the ordinary clamp formation (Section 1.3.1.2) occurring in some basidiomycetes (Rypáček 1966). Further, clamp presence, type, shape and size are important characters in cultural keys for the identification of decay fungi (Zabel & Morrell 1992). Figure 5.26 shows a medallion clamp produced by the fungus *A. sinuosa* clearly illustrating the special formation where the clamp is somewhat stretched into an oval or circle, compared to the ordinary clamp within the same picture (ordinary clamp formation in Section 1.3.1.2). Another difference between the two clamps is that for medallion clamps, the cell dividing septae can not be distinguished with the light microscope (Huckfeldt & Schmidt 2006a) with the clamp therefore appearing as single 'roundabout' hyphae. *G. sepiarium* for example is known to produce medallion clamps within its surface mycelium (Rypáček 1966) as it sometimes occurs in *Antrodia* sp. (Huckfeldt & Schmidt 2006b). Schwarze & Spycher (2005) detected medallion clamp formation for the two brown rot decay fungi *G. trabeum* and *O. placenta* in their thermo-hygro-mechanically densified *Picea abies*, an especially interesting observation as it is the only report found in the literature picturing these type of clamps for *G. trabeum*. In this PhD thesis research, no medallion clamps could be recognised within the strain of *G. trabeum* used. Further, as expected, the strain of *O. placenta* frequently produced medallion clamps within the test block, whereas they were found less often in *A. sinuosa*, as observed by Huckfeldt & Schmidt (2006b).

Many, if not all, brown rot fungi possess the ability to produce acids, mainly a variety of organic acids but particularly oxalic acid/oxalate (Jennings 1991), which can cause a drop in pH *in situ* and as measured in growth medium (Eaton & Hale 1993). A well-studied fungus producing oxalate in rather large quantities is *S. lacrymans*, able to acidify the growth medium (nutrient liquid after two months incubation) to pH 2.4 (Schmidt 1995b). This oxalate is suggested to serve as an acid catalyst for the hydrolytic breakdown of wood polysaccharides (Schmidt

2006; Section 1.5.7), attacking first the hemicelluloses and then the amorphous cellulose with the result of an increase in porosity of the wood structure, freeing the pathway for hyphae, enzymes and low-molecular mass degrading substances (Green *et al.* 1992). Generally, excess oxalic acid can be neutralised to insoluble oxalate crystals, such as calcium oxalate and copper oxalate (Vesentini *et al.* 2007), for example calcium from brickworks or chelating with ions from metals (Schmidt 2006). Clausen & Green (2003) showed that copper tolerant brown rot fungi such as *O. placenta* or *A. vaillantii* produced 2-17 times more oxalic acid in copper-citrate treated southern yellow pine species (SYP) compared to untreated controls, whereas Green *et al.* (1997) found earlier that SYP colonised and degraded by *O. placenta* showed an increase in the trace element calcium content to be two- to threefold after 4 weeks. Calcium is considered an important agent in regulating plant cell wall hydrolyses (Rihouey *et al.* 1995).

During Decay tests I and II, as described in Chapter 3, none of the four brown rot fungi tested produced observable oxalate crystals in untreated *P. radiata* control samples (Table 5.1). These crystals can possibly be directly linked with the detoxification (or in other words cation binding ability of oxalic acid) of the immediate environment around fungal hyphae (Humar *et al.* 2002; Clausen & Green 2003; Green & Clausen 2003). To not find such crystals in untreated *P. radiata* decayed wood might be because, for example, the trace element calcium was present in enough quantity to regulate the cell wall hydrolyses as had occurred in all blocks and shown microscopically, but not in excess for calcium oxalate crystals to be produced. However, as recently demonstrated by Schilling & Jellison (2006), brown rot fungi such as *F. pinicola* and *G. trabeum* exhibit the capacity to control the extracellular environment differently inside and outside the wood matrix and further showed that metal enriched wood does not necessarily lead to an increased production of oxalic acid in these two fungi or promote crystal production. For example *F. pinicola* produced Ca oxalate crystals whereas *G. trabeum* did not (Schilling & Jellison 2006), outlining the inconsistency in brown rot oxalate crystals formation.

Throughout the wood sections examined by LM and FE-SEM, a few bacteria were detected for every wood/fungus combination, but not in *P. radiata* control sections. This unexpected observation is at first sight puzzling as all precautions to avoid contamination during the decay test had been taken. The inoculating fungal cultures were free of bacteria, as demonstrated by visual observation and DNA extractions and PCR amplification being negative with bacterial primers, and decay test blocks were sealed in plastic bags and gamma irradiated prior to inoculation in gas sterilised plastic containers containing autoclaved malt agar extract. Contamination during section preparation while bringing those into contact with the two aqueous stains and deionised water was unlikely, since control sections were handled in the same manner. It is well known that bacteria are the most numerous and ubiquitous of organisms, capable of colonising wood under both aerobic and anaerobic conditions (Clausen 1996). Two aerobic explanations might be plausible to account for bacterial occurrence within the decayed blocks: a) bacterial infection was brought into test containers during inoculation or b) bacterial infection occurred after the incubation time when samples were freed from adhering mycelium and air dried. The latter seems likely as no prominent bacterial decay patterns such as erosion or tunnelling (Daniel & Nilsson 1997; Section 1.6.4) were observed during microscopy. It is therefore hypothesised that the bacteria were secondary contaminants able to use the degraded wood structure to penetrate into the wood tracheids during the drying procedure. Nevertheless, further investigations are necessary to determine if the occurrence of bacteria during or after a decay test is a common phenomenon that has not been reported in current literature.

6 Conclusions and Future Research Areas

This PhD thesis researched the causal agents of decay in leaky walls of New Zealand houses/buildings; a problem consumers were faced with particularly during the past 10 years. From more than 2000 leaky building samples were chosen 421, predominantly from untreated *Pinus radiata* decayed framing and also fibre cement boards and building paper, to investigate fungal biodiversity associated with decay. In addition, air sampling was conducted at a multi-unit building site in Auckland, which was proven to have serious decay issues; air sampling was done in interior rooms, exterior, and in wall cavities.

Sixty-eight fungal species including brown-, white- and soft-rot fungi were found to be associated with leaky buildings. Fifty-five species were identified using molecular analysis of DNA extracted from pure cultures and the BLAST search tool on GenBank, comparing rDNA sequence composition to known ITS sequences. The conservative assumption was made that >95% ITS region sequence identity was enough to confidently group taxa into a genus (Landeweert *et al.* 2003). Additionally, thirteen identifications were based on traditional morphological examinations of fruiting bodies found in leaky buildings. Further, 45% of the decay fungi isolated from leaky NZ wall cavity materials are known to occur in buildings worldwide, outlining their importance and significance in the degradation of wooden construction materials in houses. Of the fifty-five fungal species identified in the PhD thesis research, this is the first report of thirty-three decay fungi from NZ leaky buildings.

These results therefore show that there is a high biodiversity of fungi isolated from the leaky building samples, with only four brown rot decay fungi being repeatedly isolated, as follows: *Gloeophyllum sepiarium* (isolated from 3.1% of samples), *Oligoporus placenta* (isolated from 2.6% of samples), *Antrodia sinuosa* (isolated from 1.9% of samples), and *Gloeophyllum trabeum* (isolated from 1% of samples). These four fungal species have all been reported as important brown rot fungi of softwood, pine and fir wood products (Grosser 1985; Ridout 2000; Schmidt 2006). Justification that there is high biodiversity represented in the leaky building samples is that these four fungi constitute less than 10% of the fungal species and all other isolates occurred at a frequency of <1%. This is exceptionally relevant as it clearly shows that there is not just one ‘super-bug’ fungus causing the leaky building problem. With high biodiversity represented, it is clear also that solutions for wood preservative treatments should be robust and be able to prevent a wide range of fungal organisms from causing decay.

High biodiversity has been shown in decaying wood, specifically the study of Nakamura (2002), which showed Ascomycetes, Deuteromycetes, and sterility fungi, as well as 32 types of sterility fungi in decaying dead trunks of *Rhizophora stylosa* and *Bruguiera gymnorrhiza* in a mangrove forest. Dominant species of fungi involved in decomposition were different by the trunk and bark of both tree species, forest floor swamp, and waterway, which showed high biodiversity. This study, though, as most of the literature in this area, describes high biodiversity in an ecosystem setting, and not a building environment. Wei & Dai (2004) also described high biodiversity in an ecosystem and attribute high biodiversity of wood-decaying fungi as one of the important factors for the health of forest ecosystem. A future research area would be to extend the findings of biodiversity of decayed building samples versus decayed wood in ecological settings, and to understand the relevance of competition and successive colonization especially in a situation with high biodiversity.

The results of this thesis research showed that the molecular approach using PCR techniques, and here in particular the application of a nested PCR reaction using the primer pairs NSI1 + NLB4 (Martin & Rygiewicz 2005) and ITS1-F + ITS4 (White *et al.* 1990; Gardes & Bruns 1993), comprised a useful tool for identification of decay fungi present in environmental and pure culture samples. The molecular approach was the preferred identification method in this study, since fungal fruiting body occurrences were low and only 13 were found in more than 2000 leaky building samples available during the thesis research period. DNA extractions were made from established pure cultures from the leaky building samples and from the air samples, and from direct fungal DNA extractions from decayed wood samples, eight of which were done. The latter method of DNA extraction directly from wood holds potential for ‘leaky building’ fungal identifications, since today's commercial DNA extractions kits, combined with fungal specific oligonucleotide primers, do not require pure fungal cultures and therefore can provide a quick turn-around time when evaluating building conditions and indicate possible precautions that should be considered during remediation.

Moreth & Schmidt (2000) showed that the development of taxon-specific primers for common 'house'-rot fungi can be useful as a screening device, and are often used commercially in Germany, since the building surveyor, in accordance with building regulations (DIN68800 Part 4), has to determine if *Serpula lacrymans* was present or not at a specific building site (Huckfeldt & Schmidt 2006a). However, it is not always possible to develop taxon-specific primers, especially for fungi belonging to the same genera, as was shown for *Armillaria* species in the past (Potyralska *et al.* 2002). Further, unknown and as yet not sequenced fungi might share similar sequence patterns and, therefore, confirmed identifications are not always possible. Nevertheless, the technique provides a good indication to confirm or eliminate dominant or common wood decay fungi. The design of taxon-specific primers for common wood decay fungi from NZ leaky buildings in the future could aid in this regard.

Misperception of causal decay occurred at a Leaky Buildings Symposium in 2005 (18th & 19th July 2005, Auckland NZ, organised by the University of Auckland), where it was assumed that the brown rot fungus *S. lacrymans* constituted one of the biggest threats to leaky buildings in NZ, but no specific examples were given. This PhD thesis research showed, using fungal- and non-taxon- specific DNA primers to amplify DNA from cultures developed from the leaky building samples from the Auckland region where most of the leaky buildings issues persisted in the year of 2008, that *S. lacrymans* could not be detected. *S. lacrymans* was found in only one of the 421 samples examined in the thesis research and it originated from Christchurch, South Island, NZ.

It is noteworthy that DNA molecular evidence indicated the isolation and culture from NZ samples of the worldwide common building fungus *O. placenta* (Morris & McFarling 2007; Schmidt 2007; Dai & Penttilä 2006 (in nature reserve in northeastern China)). This decay fungus has not previously been recorded for NZ, but is reported to occur in Australian houses (Walters 1973). A key future area of follow-up research would be to confirm *O. placenta* is in New Zealand and is one of the causative agents of decay in leaky buildings. This is suggested to be done by using additionally DNA primers to further confirm the identity of the PhD thesis research cultures as *O. placenta*, and to do a phylogenetic analyses of them as compared to other southern and northern Hemisphere isolates.

Mycologists have studied the fungal diversity in NZ since the late 19th Century; however, the number of fungal species present in NZ is expected to exceed 20,000, with probably three quarters remaining to be recorded (Gadgil 2005). As *O. placenta* is globally reported, it can be suggested that the most likely explanation for this first detection, and it being in leaky NZ houses, lies in the limited studies into decayed building structures that have been conducted, NZ's high number of unknown fungal species or possibly a recent introduction of the fungus to the country, perhaps within the last decade since the leaky buildings problems have been most noted in the last decade. Therefore, it would be highly interesting to find a specimen of *O. placenta* in, for example, a forest environment, especially because so far no distinctive fruiting body has been found.

This study also examined the likelihood of detection of viable aerial fungal spores of decay fungi. It was proven for a multi-unit leaky building complex in Auckland that viable fungal aerial spores were present inside, outside and within leaky wall cavities, with highest numbers being detected in water-damaged walls. This finding clearly illustrated that leaky walls are susceptible to fungal spore accumulation, which can indicate not only a potential moisture-related problem within a particular wall, but as well the risk of decayed framing. From the aerial spore study, pure cultures were developed and these identified by molecular analysis. Indoor and outdoor air sampling is commonly used in NZ (Waipara 2002 and 2003) to determine spore concentrations (viable and non-viable) with respect to potential health hazards to both flora and fauna, including humans. The additional sampling of wall cavity air in the future has the potential for early detection of a potential decay hazard, prior to any costly destructive visual examinations of the building fabric. Nevertheless, visual examinations become a necessity if framing deterioration is suspected, and further microscopic and/or molecular studies to be definitive in evaluating wood colonisation by decay fungi (Clausen & Kartal 2003).

A fifth yet unknown fungus was found in this study in 8 of the 421 samples (1.9% occurrence) and should be investigated in the future to establish the extent of its degradation of softwood framing in leaky NZ buildings. This fungus, with a confidence level of 85%, showed homology to *Pycnoporellus fulgens*, a brown rot not reported from NZ but which is thought to be closely related to the Australasian fungus *Rigidoporus laetus* (Hood 1992) and endemic to NZ but of which no sequence was available in the GenBank databases. For this reason, it would be highly interesting to do a phylogenetic study of NZ isolates of *R. laetus* and compare these to the unknown cultures from this study.

Further, the study of the evolutionary connections between the most common NZ leaky building decay fungi and those isolated in the USA and Europe has great potential to, hypothetically, outline their similarity genetically and to determine the mechanism(s) of degradation of the building materials. Future suggested international standard test organisms in laboratory decay trials may utilize the NZ isolates which could also be used to further study and to optimise wood preservation chemicals.

This thesis research presented data that showed well-defined dosage responses and approximate toxic thresholds for wood preservatives to elucidate the effectiveness of wood preservatives approved for NZ. The following should provide valuable points of reference for future decay test methodology and refinement of preservative treatments, as follows:

1. The isolated test fungus *Antrodia sinuosa* was more difficult to control with tebuconazole plus propiconazole (1:1) at retention 0.007% m/m than the known tolerant fungus *Oligoporus placenta*;

2. Boron at the highest retention tested of 0.4% m/m boric acid equivalent (BAE) as specified by Hazard Class 1.2 of NZS3640:2003 was not toxic to *Oligoporus placenta*;
3. *Serpula lacrymans* exhibited tolerance to the highest tested retention of 0.06% m/m tebuconazole plus propiconazole (1:1), but was susceptible towards the CuAz treatment; since some strains of *S. lacrymans* exhibit Cu tolerance (Hastrup *et al.* 2005), future tests on Cu sensitivity of the fungal isolate used in this study are suggested;
4. *Gloeophyllum* species appeared susceptible to all wood preservatives;
5. Minimum IPBC retention as specified by Hazard Class 1.2 of NZS3640:2003 (0.025% m/m) is on the low side; and
6. Complete loss of efficacy of 0.4% m/m BAE after the 2 week leaching regime.

The laboratory decay tests in this study showed that untreated and wood preservative treated framing timber were susceptible to all the tested decay fungi, outlining their importance and capability of wood biodegradation. The results of the thesis research suggested that especially houses where untreated framing timber was used in the past in NZ are more likely to be at risk, especially if moisture related problems prevailed for a long period of time. This does not imply that treated framing would not also be at risk under the same wet conditions, as, for example, it was shown for boron treated framing and for IPBC treated framing to be susceptible after a 2 week leaching regime resulting in degradation of the test blocks.

Most biocides are designed to protect the wood against a broad range of organisms during its intended service life (Schultz *et al.* 2007) and the four treatments tested were very effective in the prevention of wood degradation by the two *Gloeophyllum* species. To ensure buildings are more protected from decay organisms in the future, the preventative procedures that need to be employed to adequately protect the framing structure are as follows:

1. the application of correct design and construction principles and,
2. depending on the intended use, such as exterior walls with the risk of exposure to moisture, preservative treated framing.

The procedures suggested to be considered in the future during building remediation have the same values as preventative procedures and should include the following determinations, considerations and actions:

- Type of decay, being dead or inactive infestation or alive and active decay (e.g. through vital staining with fluorescein diacetate (FDA) (Huckfeldt *et al.* 2000)) and main causative decay fungal identification where possible;
- The original cause(s) and extent of damage to the building fabric;
- Dryness of affected timbers and capability to keep them dry (MC ≤ 20%);
- Replacement of degraded framing with adequately protected framing for the intended in-situ use, to minimise the risk of re-infection e.g. if *O. placenta* had been the causative decay fungus in a leaky building, replacement framing could include an azole treatment instead of boron, since this fungus exhibited some tolerance towards boron in this study at its NZS3640:2003 specified retention of 0.4% m/m, but it could be used if *Gloeophyllum* species were the main decay agents;
- Building repairs to establish the correct construction and design principles to shield the framing from weathering and decay in the future.

However, exigency and extent of a leaky building remediation should be carefully balanced to avoid unnecessary use of chemicals that could have an adverse effect on the homeowner (e.g. indoor air quality) and the environment (e.g. chemical leaching into the surrounding soil), and other building material replacement and reconstruction related costs.

Microscopic studies provided the baseline for evaluation of morphological changes in wood (Wilcox 1993a). The correlative light microscopic and field-emission scanning electron microscopic investigation in this thesis research proved complementary and allowed for more precise analyses and interpretation of key findings as observed for untreated *P. radiata* degraded wood blocks following a standard twelve weeks laboratory decay test. Ray parenchyma cells were colonised at early stages of decay but not necessarily degraded since intact cells were present throughout late stages of decay, suggesting that they are not preferentially degraded early in the brown rot decay process, as previously indicated by Schwarze (2007). Even though the presence of hyphae in wood confirmed colonisation, their distribution within wood rays and tracheids gave little information about the advancement of decay as given by mass loss data. The presence of fine (≤ 1µm) to wide (≥ 3.5µm) bore-hole and hyphal size ranges, and nearly unchanged cell wall thicknesses of all wood/test fungi combinations, confirmed active decay at moderate to late stages. Additionally, the presence of bore-holes, clamp and medallion clamp formation and resting spores (chlamydo- and arthrospores) are fungal specific, aided in fungal differentiation and identification, and should be recorded during wood decay studies, as resting spores especially are an important factor when planning remediation strategies.

The picro aniline blue stain had a tendency to dye wood cell wall material at later stages of decay, obstructing fungal hyphae features close to the luminal layer. This problem might be overcome if this stain is used in combination with the counter-stain Safranin 0 (Wilcox 1964), which highlights the cell wall in more detail.

Some conclusions regarding the decay fungi studied are as follows:

- The brown rot fungus *Antrodia sinuosa* showed the greatest variability in bore-hole sizes, caused the highest mass loss in untreated *P. radiata* and showed early signs of cell wall thinning.
- *Serpula lacrymans* and *Gloeophyllum trabeum* hyphae were most abundant in wood rays at an early mass loss range of 10-15%, supporting the primary pathway of wood colonisation by brown rot fungi.
- Only the microscopic observations coupled with mass loss data could reveal the extent of degradation in *G. trabeum* colonised *P. radiata* blocks, outlining the difficulties of detecting this fungus during early stages of decay in e.g. construction timbers.

- *S. lacrymans* and *Oligoporus placenta* hyphae illustrated that brown rot fungi can target apical growth towards degraded pit apertures, emphasising their ability to locate open pathways to colonise wood.
- Relying on birefringence as a tool to record changes in cell wall crystallinity associated with brown rot decay alone could be misleading, since with *P. radiata* colonised by the decay fungus *G. trabeum*, the orientation of the section (tangential/radial/transverse) or the thickness of the sections can impair this observation.
- *A. sinuosa*, *O. placenta* and *G. trabeum* produced chlamydospores whereas *S. lacrymans* produced arthrospores within the wood matrix, a fungal specific decay feature previously only recorded in pure culture identification keys (Stalpers 1978). However, one identification key to strand forming fungi in houses (Huckfeldt & Schmidt 2006b) describes the presence of these spore types for some of their decay fungal species.

Bacteria were found as contaminants in all wood/fungi combinations but not in controls, and were not found in the fungal cultures used for inoculation. It was suggested that they were possibly introduced after the decay test, during the drying step. Since no reports were found in current literature of the likelihood of bacteria occurring during or after a standardised decay test, this makes further investigations necessary to determine what was the cause of the contamination.

This microscopic investigation of morphological changes in untreated *P. radiata,* caused by the NZ common leaky building fungi *Antrodia sinuosa*, *Oligoporus placenta* and *Gloeophyllum trabeum,* outlined how important the evaluation of the extent of decay is for the determination of recommendations in leaky buildings remediation, especially for the early stages where degradation can have occurred inside the construction timber with no obvious signs such as mycelium on the timber's surface. A highly interesting follow-up study from this thesis research would be the examination of wood preservative and beech control samples to determine any variances in decay morphology.

In Summary, this PhD thesis research provided the first comprehensive investigation into the biodiversity of decay fungi from leaky New Zealand buildings, and presented details about their wood decay micromorphology. From the samples studied, there appears to be high biodiversity in New Zealand leaky buildings and therefore prevention must be broad enough to be effective against this diverse range of fungal organisms. It also demonstrated substantial differences in efficacy of preservative formulations currently approved for framing treatments in New Zealand and possible deficiencies where framing may be subjected to severe leaching. This study also provided the first comparative look at viable fungal aerial spores between leaky wall cavities and the surrounding air environment. Subsequently, this research added to the knowledge of the decay fungal species diversity in and around New Zealand leaky buildings, outlined their capabilities to degrade treated and un-treated *P. radiata* framing timber and illustrated the efficacy of New Zealand approved wood preservatives for their potential as remedial treatment and future prevention.

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Appendix 1

The following unnumbered pages present the two papers from this PhD thesis research, which were published by the *International Research Group on Wood Protection* in the years of 2007 and 2008:

Document IRG/WP 07-10620

Wood decay fungi from New Zealand 'leaky' buildings:
PCR identification and laboratory decay tests of wood preservative-treated *Pinus radiata* (Part 1)

Dirk Stahlhut, Roberta L. Farrell, Robin Wakeling, Mick Hedley

and

Document IRG/WP 08-10649

Wood decay fungi from New Zealand leaky buildings
- PCR identification (Part 2) and aerial spore trapping

Dirk Stahlhut, Roberta L. Farrell, Robin Wakeling, Mick Hedley

Appendix 2

2.1 Treatment calculation

Treatment calculations were performed following the instructions supplied by the preservative concentrate manufacturer Koppers Arch Ltd. Auckland, NZ, based on a treatment solution of 4l to achieve the required highest retention. The needed treatment solution was based on the treating vessel, predicted uptake per block and wood volume within the vessel. A test run with 20 wood blocks each for the two treatment carrier systems predicted the following uptakes in litre per cubic meter:

Water as carrier: **600l/m^3**

Solvent (PegasolAA) as carrier: **580l/m^3**

The wood block size was **35x35x7 mm** with a volume of **0.000008575m^3** and 192 blocks were needed for each of the two lowest retentions and treatment, and 240 blocks were required for each of the highest retention. Wood volume (in ml) per treatment charge were as follows:

192 blocks= **1646ml**

240 blocks= **2058ml**

The calculated preservative concentrate in millilitre per 4 litre were as follows:

Retentions:	0.4%	0.13%	0.04%
Boron treatment			
Diffusol			
Concentrate (ml)/4l	30	9.75	3
Tan E (CuAz)	0.3%	0.1%	0.03%
Concentrate (ml)/4l	69.6	23.2	6.96
IPBC	0.1%	0.03%	0.01%
Concentrate (ml)/4l	50	15	5
Azole Teb/Prop (1:1)	0.06%	0.02%	0.007%
Concentrate (ml)/4l	40	13.33	4.67

Treatments were carried out in the following order:

Treat order	Treatment	Predicted uptake (l/m^3)	Mix volume (ml)	Solution surplus (ml)	Preservative add (ml)	Container volume (ml)/cm	Wood volume (ml)	Uptake (ml)	Depth (cm)	Void volume (ml)	Min volume (ml)
1	boron0.04	600	4000	3000	3.0	676	1646	1000	7	3,086	4,086
2	boron0.13	600	4000	3012	7.5	676	1646	988	7	3,086	4,073
3	boron0.4	600	4000	2694	22.7	676	2058	1306	7	2,674	3,980
4	CuAz0.03	600	4000	3000	7.0	676	1646	1000	7	3086	4086
5	CuAz0.1	600	4000	3000	18.0	676	1646	1000	7	3086	4086
6	CuAz0.3	600	4000	2738	52.2	676	2058	1262	7	2674	3936
7	IPBC0.01	580	4000	3045	5.0	676	1646	955	7	3086	4041
8	IPBC0.03	580	4000	3045	11.2	676	1646	955	7	3086	4041
9	IPBC0.1	580	4000	2806	38.6	676	2058	1194	7	2674	3868
10	Teb/Prop0.007	580	4000	3045	4.7	676	1646	955	7	3086	4041
11	Teb/Prop0.02	580	4000	3045	9.8	676	1646	955	7	3086	4041
12	Teb/Prop0.06	580	4000	2806	29.9	676	2058	1194	7	2674	3868

2.2 Mass loss chart data

The following two tables are to assist explanatory to read the coding of the mass loss results.

Treatment	**Index**	**Leached for two weeks**
Boron	B	BL, K, L, M, N, O, P
CuAz	C	CL, K, L, M, N, O, P
IPBC	I	IL, K, L, M, N, O, P
Azole	CA	CAL, K, L, M, N, O, P
Pine	R	
Water	W	
Solvent	S	
Beech	Q	

Trial ID numbers	**Test fungi**
A, G, M	*Oligoporus placenta*
B, J, P	*Coniophora puteana*
C, F, L	*Antrodia sinuosa*
D, H, N	*Gloeophyllum sepiarium*
I, O	*Gloeophyllum trabeum*
E, K	*Serpula lacrymans*

		Average	STDEV		Average	STDEV
	A-B0	5.24	1.83	A-I0	-0.30	1.15
	A-B1	46.43	6.04	A-I1	6.79	3.61
	A-B2	55.23	5.11	A-I2	35.40	9.52
	B-B0	2.04	0.24	B-I0	3.05	4.21
	B-B1	2.01	0.43	B-I1	24.41	8.45
	B-B2	31.01	7.21	B-I2	41.01	5.07
	C-B0	0.83	0.42	C-I0	-0.83	1.41
	C-B1	4.79	5.32	C-I1	18.79	8.42
	C-B2	31.02	16.78	C-I2	47.14	11.76
	D-B0	0.59	0.32	D-I0	-1.01	0.95
	D-B1	1.75	1.65	D-I1	-1.22	0.49
	D-B2	6.77	3.40	D-I2	4.08	4.46
	E-B0	-0.25	0.18	E-I0	-1.69	0.48
	E-B1	0.22	0.62	E-I1	3.29	4.20
	E-B2	7.21	3.31	E-I2	9.18	4.82
	F-B0	-0.42	1.10	F-I0	-2.37	0.90
	F-B1	1.65	2.63	F-I1	10.50	9.46
	F-B2	18.14	9.95	F-I2	30.53	17.00
	G-B0	2.24	3.35	G-I0	-0.68	0.60
	G-B1	32.50	8.96	G-I1	5.13	5.91
	G-B2	34.35	7.86	G-I2	29.09	9.53
	H-B0	-0.12	0.30	H-I0	-2.15	0.51
	H-B1	2.75	2.25	H-I1	-1.97	0.88
	H-B2	34.91	3.26	H-I2	18.24	5.26
	I-B0	-0.06	0.14	I-I0	-1.99	0.39
	I-B1	-0.51	0.19	I-I1	1.62	4.36
	I-B2	15.88	4.86	I-I2	13.35	4.04
	J-B0	0.07	0.21	J-I0	6.15	6.22
	J-B1	-0.23	0.28	J-I1	23.27	9.23
	J-B2	24.40	5.78	J-I2	34.76	4.35
Leached	A-BL	52.91	5.07	A-IL	3.30	2.05
	B-BL	40.72	5.49	B-IL	13.64	5.87
	C-BL	62.58	3.79	C-IL	14.46	4.82
	D-BL	18.93	3.68	D-IL	-0.52	0.45
	K-B0	19.51	4.54	K-I0	9.11	5.02
	L-B0	57.73	5.71	L-I0	21.19	12.32
	M-B0	35.63	8.45	M-I0	12.41	5.14
	N-B0	30.30	2.90	N-I0	4.48	4.30
	O-B0	14.15	2.56	O-I0	10.89	5.98
	P-B0	32.84	3.37	P-I0	26.38	6.01

		Average	STDEV		Average	STDEV
	A-CA0	0.35	0.83	A-C0	2.87	0.93
	A-CA1	4.05	3.01	A-C1	25.88	9.01
	A-CA2	22.28	3.96	A-C2	53.55	5.42
	B-CA0	0.01	0.43	B-C0	1.95	0.61
	B-CA1	2.22	2.68	B-C1	8.03	7.46
	B-CA2	10.63	7.36	B-C2	37.52	7.67
	C-CA0	-0.12	0.75	C-C0	0.76	0.50
	C-CA1	12.58	4.79	C-C1	10.21	7.05
	C-CA2	44.85	6.14	C-C2	56.76	3.92
	D-CA0	-0.97	0.48	D-C0	0.37	0.41
	D-CA1	-0.86	0.64	D-C1	0.41	0.52
	D-CA2	-0.18	1.04	D-C2	2.57	1.23
	E-CA0	5.43	3.35	E-C0	0.02	0.30
	E-CA1	8.31	3.86	E-C1	4.78	3.71
	E-CA2	13.09	6.71	E-C2	18.59	4.05
	F-CA0	-1.83	0.36	F-C0	-0.61	0.25
	F-CA1	1.52	4.28	F-C1	2.09	3.97
	F-CA2	47.44	15.16	F-C2	55.22	5.30
	G-CA0	-1.49	0.41	G-C0	2.09	1.77
	G-CA1	1.23	1.90	G-C1	31.60	8.59
	G-CA2	21.80	4.78	G-C2	41.82	7.52
	H-CA0	-2.10	0.54	H-C0	-0.04	0.19
	H-CA1	-1.18	0.46	H-C1	0.00	0.28
	H-CA2	-1.02	0.27	H-C2	5.43	4.44
	I-CA0	-1.52	0.49	I-C0	-0.21	0.18
	I-CA1	-1.24	0.77	I-C1	-0.22	0.21
	I-CA2	-0.77	0.69	I-C2	0.93	0.62
	J-CA0	-0.80	1.03	J-C0	-0.21	0.51
	J-CA1	5.31	3.18	J-C1	5.98	7.76
	J-CA2	18.89	8.24	J-C2	35.88	4.01
Leached	A-CAL	1.18	0.55	A-CL	4.47	1.56
	B-CAL	1.10	0.41	B-CL	0.96	0.67
	C-CAL	1.22	0.65	C-CL	9.31	8.83
	D-CAL	-0.02	0.67	D-CL	0.17	0.41
	K-CA0	7.44	4.54	K-C0	14.07	5.29
	L-CA0	-1.44	0.56	L-C0	0.26	4.65
	M-CA0	-0.98	0.33	M-C0	-0.45	1.63
	N-CA0	-1.10	0.26	N-C0	-1.33	0.28
	O-CA0	-0.80	0.20	O-C0	-0.99	0.16
	P-CA0	-0.88	0.58	P-C0	-1.13	0.27

Appendix 3

3.1 Colony forming units

The following presents the actual colonies formed on each plate (**R**) along the amount of air sampled i.e. R50 = 50 litre of air, the corrected value (**Pr**) after Feller (1950) and the colony forming units per cubic meter (**CFU/m^3**) for each sampling location.

Location B- 2.5m above ground at open stud outside

Media:	R50	Pr50	CFU/m3	Average	STDV	Sample Order	R200	Pr200	CFU/m3	Average2	STDV2	Sample Order2
CMC	17	17	340	320	28	3	165	212	1060	695	516	6
	15	15	300			4	61	66	330			7
BMA	1	1	20	20	0	5	4	4	20	25	7	1
	1	1	20			6	6	6	30			8
YMA	31	32	640	630	14	1	92	104	520	450	99	3
	30	31	620			2	69	76	380			5
MEA	23	24	480	810	467	7	88	99	495	485	14	2
	53	57	1140			8	85	95	475			4

Location BI- 1m away on ground level outside

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R200	Pr200	CFU/m3	Average2	STDV2	Order2
CMC	14	14	280	290	14	5	44	47	235	237.5	4	6
	15	15	300			6	45	48	240			7
BMA	2	2	40	20	28	3	16	16	80	55	35	4
	0	0	0			4	6	6	30			5
YMA	42	44	880	790	127	7	61	66	330	297.5	46	1
	34	35	700			8	50	53	265			8
MEA	19	19	380	360	28	1	54	58	290	242.5	67	2
	17	17	340			2	37	39	195			3

Location C- inside wall cavity 2.5m above ground corner outside wall

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R200	Pr200	CFU/m3	Average2	STDV2	Order2
CMC	12	12	240	690	636	6	92	104	520	587.5	95	6
	53	57	1140			7	112	131	655			7
BMA	12	12	240	170	99	4	74	82	410	245	233	4
	5	5	100			5	16	16	80			5
YMA	179	237	4740	3470	1796	1	316	622	3110	2115	1407	1
	96	110	2200			8	172	224	1120			8
MEA	91	103	2060	4280	3140	2	297	541	2705	5640	4151	2
	223	325	6500			3	395	1715	8575			3

Location CI- 1m away from corner wall ground level

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R200	Pr200	CFU/m3	Average2	STDV2	Order2
CMC	7	7	140	170	42	3	42	44	220	210	14	5
	10	10	200			4	38	40	200			6
BMA	4	4	80	50	42	5	7	7	35	30	7	3
	1	1	20			6	5	5	25			4
YMA	20	20	400	400	0	1	43	45	225	255	42	7
	20	20	400			2	53	57	285			8
MEA	18	18	360	320	57	7	49	52	260	297.5	53	1
	14	14	280			8	62	67	335			2

Location D- Downstairs centre of room 2m away from outside wall

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R200	Pr200	CFU/m3	Average2	STDV2	Order2
CMC	23	24	480	420	85	5	66	72	360	325	49	5
	18	18	360			6	54	58	290			6
BMA	3	3	60	50	14	3	5	5	25	35	14	3
	2	2	40			4	9	9	45			4
YMA	33	34	680	900	311	7	38	40	200	252.5	74	7
	52	56	1120			8	57	61	305			8
MEA	18	18	360	410	71	1	86	97	485	502.5	25	1
	22	23	460			2	92	104	520			2

Location DI- Upstairs same centre location as downstairs 2m from worst wall

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R200	Pr200	CFU/m3	Average2	STDV2	Order2
CMC	17	17	340	280	85	5	39	41	205	207.5	4	5
	11	11	220			6	40	42	210			6
BMA	1	1	20	20	0	3	9	9	45	47.5	4	3
	1	1	20			4	10	10	50			4
YMA	13	13	260	370	156	7	53	57	285	310	35	7
	23	24	480			8	62	67	335			8
MEA	23	24	480	530	71	1	79	88	440	397.5	60	1
	28	29	580			2	65	71	355			2

Location E- Downstairs centre of room 2m away from outside wall

Media:	R10	Pr10	CFU/m3	Average	STDV	Order	R50	Pr50	CFU/m3	Average2	STDV2	Order2
CMC	6	6	600	700	141	5	25	26	520	460	85	5
	8	8	800			6	20	20	400			6
BMA	1	1	100	100	0	3	4	4	80	60	28	3
	1	1	100			4	2	2	40			4
YMA	8	8	800	750	71	7	22	23	460	290	240	7
	7	7	700			8	6	6	120			8
MEA	16	16	1600	1350	354	1	26	27	540	470	99	1
	11	11	1100			2	20	20	400			2

Location EI- Upstairs centre location 2m away from outside wall

Media:	R10	Pr10	CFU/m3	Average	STDV	Order	R50	Pr50	CFU/m3	Average2	STDV2	Order2
CMC	5	5	500	400	141	5	19	19	380	370	14	5
	3	3	300			6	18	18	360			6
BMA	0	0	0	50	71	3	5	5	100	80	28	3
	1	1	100			4	3	3	60			4
YMA	17	17	1700	900	1131	7	17	17	340	340	0	7
	1	1	100			8	17	17	340			8
MEA	13	13	1300	950	495	1	36	38	760	640	170	1
	6	6	600			2	25	26	520			2

Location F- outer corner 1.7m above ground out of stud cavity outside

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R100	Pr100	CFU/m3	Average2	STDV2	Order2
CMC	37	39	780	810	42	5	49	52	520	610	127	5
	40	42	840			6	64	70	700			6
BMA	1	1	20	70	71	3	11	11	110	90	28	3
	6	6	120			4	7	7	70			4
YMA	19	19	380	990	863	7	259	416	4160	2865	1831	7
	73	80	1600			8	130	157	1570			8
MEA	130	157	3140	2710	608	1	321	647	6470	6950	679	1
	99	114	2280			2	338	743	7430			2

Location FI- 1m away from decayed corner stud 50cm above ground level

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R100	Pr100	CFU/m3	Average2	STDV2	Order2
CMC	35	37	740	590	212	5	36	38	380	405	35	5
	21	22	440			6	41	43	430			6
BMA	2	2	40	20	28	3	0	0	0	0	0	3
	0	0	0			4	0	0	0			4
YMA	17	17	340	490	212	7	38	40	400	490	127	7
	31	32	640			8	54	58	580			8
MEA	30	31	620	720	141	1	42	44	440	415	35	1
	39	41	820			2	37	39	390			2

Location G- inner Unit, outer Stud sample inside wall cavity 1.8m above ground

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R100	Pr100	CFU/m3	Average2	STDV2	Order2
CMC	20	20	400	330	99	5	44	47	470	495	35	5
	13	13	260			6	49	52	520			6
BMA	11	11	220	230	14	3	24	25	250	225	35	3
	12	12	240			4	20	20	200			4
YMA	29	30	600	630	42	7	71	78	780	720	85	7
	32	33	660			8	61	66	660			8
MEA	51	54	1080	1100	28	1	65	71	710	1605	1266	1
	52	56	1120			2	186	250	2500			2

Location GI- sample 1m away from corner stud 50cm above ground outside

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R100	Pr100	CFU/m3	Average2	STDV2	Order2
CMC	12	12	240	400	226	5	37	39	390	290	141	5
	27	28	560			6	19	19	190			6
BMA	0	0	0	10	14	3	1	1	10	5	7	3
	1	1	20			4	0	0	0			4
YMA	18	18	360	350	14	7	37	39	390	405	21	7
	17	17	340			8	40	42	420			8
MEA	24	25	500	470	42	1	54	58	580	455	177	1
	21	22	440			2	32	33	330			2

Location H- Centre of room sample downstairs

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R100	Pr100	CFU/m3	Average2	STDV2	Order2
CMC	17	17	340	330	14	5	39	41	410	405	7	5
	16	16	320			6	38	40	400			6
BMA	1	1	20	20	0	3	0	0	0	0	0	3
	1	1	20			4	0	0	0			4
YMA	28	29	580	610	42	7	58	63	630	610	28	7
	31	32	640			8	55	59	590			8
MEA	19	19	380	420	57	1	32	33	330	315	21	1
	22	23	460			2	29	30	300			2

Location HI- Centre of room upstairs exact location as downstairs 2m away from walls

Media:	R50	Pr50	CFU/m3	Average	STDV	Order	R100	Pr100	CFU/m3	Average2	STDV2	Order2
CMC	12	12	240	260	28	5	23	24	240	250	14	5
	14	14	280			6	25	26	260			6
BMA	3	3	60	40	28	3	1	1	10	5	7	3
	1	1	20			4	0	0	0			4
YMA	15	15	300	390	127	7	65	71	710	625	120	7
	23	24	480			8	51	54	540			8
MEA	30	31	620	590	42	1	33	34	340	395	78	1
	27	28	560			2	43	45	450			2

3.2 CMC positives

Location	CMC positives	Colony diameter	Clearing Zone	RA	R	% positives
B 200-6	0			0	165	0
B 200-7	1	15	6	0.4	61	1.6
B 50-3	2	4	3	0.8	17	11.8
		5	3	0.6		
B 50-4	5	10	3	0.3	15	33.3
		12	6	0.5		
		6	1	0.2		
		7	3	0.4		
		5	2	0.4		
B 200-I-6	7	9.5	4	0.4	44	15.9
		4	2	0.5		
		2.5	2	0.8		
		2.5	2	0.8		
		2.5	2	0.8		
		2.5	2	0.8		
		2.5	2	0.8		
B 200-I-7	2	2.5	1	0.4	45	4.4
		4	2	0.5		
B 50-I-5	1	7	4	0.6	14	7.1
B 50-I-6	3	4	2	0.5	15	20.0
		5	2	0.4		
		8	3	0.4		
C 200-5	4	2.5	2	0.8	112	3.6
		2.5	2	0.8		
		3	3	1.0		
		3	2	0.7		
C 200-6	5	2	1	0.5	92	5.4
		2.5	2	0.8		
		3.5	2.5	0.7		
		3	2.5	0.8		
		4	3	0.8		
C 50-6	2	10	6	0.6	53	3.8
		7	4	0.6		
C 50-7	0			0	12	0.0
C 200-I-6	6	2	1	0.5	38	15.8
		2	1	0.5		
		3.5	2	0.6		
		3	2	0.7		
		4	2	0.5		
		4	2	0.5		
C 200-I-5	5	4	3	0.8	42	11.9
		4.5	2	0.4		
		6	2.5	0.4		
		5.5	2.5	0.5		
		8	4	0.5		
C 50-I-3	3	15	3	0.2	7	42.9
		4	1.5	0.4		
		3.5	1	0.3		
C 50-I-4	4	6	4	0.7	10	40.0

Location	CMC positives	Colony diameter	Clearing Zone	RA	R	% positives
		4	3	0.8		
		10	4	0.4		
		6	3	0.5		
D 200-5	1	5	3	0.6	66	1.5
D 200-6	2	4	3	0.8	54	3.7
		3.5	4	1.1		
D 50-5	0			0.0	23	0.0
D 50-6	0			0.0	18	0.0
D 200-I-5	4	6	3	0.5	39	10.3
		6	2.5	0.4		
		3	2	0.7		
		3	1.5	0.5		
D 200-I-6	0			0	40	0.0
D 50-I-5	1	12	4	0.3	17	5.9
D 50-I-6	0			0.0	11	0.0
E 10-5	2	11	3	0.3	6	33.3
		7	2	0.3		
E 10-6	0			0.0	8	0
E 50-5	5	6	2	0.3	25	20.0
		6	2	0.3		
		3	1	0.3		
		5	2	0.4		
		5	1.5	0.3		
E 50-6	4	5	3	0.6	20	20.0
		4	2	0.5		
		3	2	0.7		
		6	3	0.5		
E 10-I-5	4	5.5	2	0.4	5	80.0
		9	3	0.3		
		10	4	0.4		
		17	4	0.2		
E 10-I-6	2	6	3	0.5	3	66.7
		6	4	0.7		
E 50-I-5	2	5	4	0.8	19	10.5
		7	4	0.6		
E 50-I-6	8	4	2	0.5	18	44.4
		5	4	0.8		
		6.5	2	0.3		
		4	2.5	0.6		
		4	3	0.8		
		4	3	0.8		
		4	3	0.8		
		4	2	0.5		
F 100-5	0			0	49	0
F 100-6	0			0	64	0
F 50-5	0			0	37	0
F 50-6	3	4	3	0.8	40	7.5
		3	2	0.7		
		2.5	1	0.4		
F 100-I-5	3	3.5	1	0.3	36	8.3
		5	3.5	0.7		
		2.5	1	0.4		

Location	CMC positives	Colony diameter	Clearing Zone	RA	R	% positives
F 100-I-6	2	2	1.5	0.8	41	4.9
		3.5	3	0.9		
F 50-I-5	2	12.5	4	0.3	35	5.7
		3	2	0.7		
F 50-I-6	1	7.5	4	0.5	21	4.8
G 100-5	3	3	1	0.3	44	6.8
		4	1.5	0.4		
		6	3	0.5		
G 100-6	0			0	49	0.0
G 50-5	0			0	20	0.0
G 50-6	0			0	13	0.0
G 100-I-5	0			0	37	0.0
G 100-I-6	5	3.5	3	0.9	19	26.3
		4	2	0.5		
		4	2	0.5		
		5	3	0.6		
		5.5	3	0.5		
G 50-I-5	2	24	6	0.3	12	16.7
		14	4	0.3		
G 50-I-6	0			0	27	0.0
H 100-5	6	3	1	0.3	39	15.4
		7	2	0.3		
		5	2	0.4		
		4	2	0.5		
		8	3	0.4		
		3.5	2	0.6		
H 100-6	7	6.5	3	0.5	38	18.4
		7	3	0.4		
		6	2	0.3		
		5.5	1	0.2		
		7.5	2	0.3		
		6	1	0.2		
		5.5	1	0.2		
H 50-5	8	5	1	0.2	17	47.1
		4	2	0.5		
		3	1	0.3		
		4.5	2	0.4		
		11	4	0.4		
		6	2	0.3		
		6	2	0.3		
		12	3	0.3		
H 50-6	4	5	2	0.4	16	25.0
		7	4	0.6		
		4	2	0.5		
		8	4	0.5		
H 100-I-5	4	5.5	2	0.4	23	17.4
		5	2	0.4		
		5.5	2	0.4		
		7	3	0.4		
H 100-I-6	0			0	25	0.0

Location	CMC positives	Colony diameter	Clearing Zone	RA	R	% positives
H 50-I-5	5	3	1	0.3	12	41.7
		8	3	0.4		
		5	2	0.4		
		4	2	0.5		
		4.5	2	0.4		
H 50-I-6	3	5	3	0.6	14	21.4
		6	2	0.3		
		7	3	0.4		

Appendix 4

Cell wall thickness measurements

Measurement Pine Control	Scale conversion	Average (μm)	S.D.
6	7		
7	8		
7	8		
6	7		
7	8		
8	9		
5	6		
6	7		
6	7		
6	7		
7	8		
8	9		
1.5	10	7.4	1.5
1	6		
1	6		
1	6		
1	6		
1.8	12		
1	6		
1.2	8		
0.9	6		
1.1	7		
0.8	5		
0.8	5		
1.1	7		

Measurement Pine/ *G. trabeum*	Scale conversion	Average (μm)	S.D.
12	9		
7	5		
9	7		
12	9		
12	9		
11	8		
4	7		
4	7		
3.5	6		
4.5	8		
2	3		
4	7	7.1	1.6
2.5	4		
4	7		
4.5	8		
4	7		
3	8		
2.5	6		
2.5	6		
3.54	9		
3.5	9		
3	8		
2	5		
3	8		
4	10		

Measurement Pine/ *S. lacrymans*	Scale conversion	Average (μm)	S.D.
3	8		
2.5	6		
3.5	9		
4	10		
3.5	9		
3	8		
2.5	6		
3	8		
3	8		
4.5	11		
4	10		
3	8		
3	8	7.7	1.5
3.5	9		
2	5		
3	8		
2.5	6		
3	8		
3	8		
2.5	6		
3	8		
2.5	6		
4	10		
3	8		
2	5		

Measurements Pine/ *A. sinuosa*	**Scale conversion**	**Average (µm)**	**S.D.**
2.5	6		
2	5		
2.5	6		
3	8		
2.5	6		
3	8		
3	8		
2	5		
2.5	6		
3	8		
2	5		
2.5	6	6.4	1.5
2.5	6		
3	8		
2	5		
1.5	4		
1.5	4		
2	5		
3	8		
3	8		
1.5	4		
3	8		
3	8		
3	8		
4	10		

Measurements Pine/ *O. placenta*	**Scale conversion**	**Average (µm)**	**S.D.**
5.5	8.5		
5	7.7		
4.54	7.0		
5	7.7		
3	4.6		
3.5	5.4		
5	7.7		
6	9.2		
3.5	5.4		
4	6.2		
7	8.2		
6.5	7.6		
8	9.4	7.6	1.5
7.5	8.8		
6	7.1		
9	10.6		
3	7.5		
3.5	8.8		
3.5	8.8		
3	7.5		
3	7.5		
3.5	8.8		
3.5	8.8		
3	7.5		
2	5.0		