FUNGI IN LOWLAND RIVER FLOODPLAIN ECOSYSTEMS

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Frontispiece: Sexual reproductive structures of an aquatic Oomycete growing on *Eucalyptus camaldulensis* leaves submerged in a laboratory mesocosm.
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Abstract

Fungal decomposition processes are recognised for their importance in terrestrial ecosystems, but their role in aquatic ecosystems is not as well established. In particular, the function of fungi in wetland carbon cycles is poorly understood. Indeed, one of the few studies of fungi in Australian wetlands suggested that fungi played no role in carbon cycling, based on an absence of fungal phospholipid biomarkers in sediments. In this thesis I examine the community dynamics of aquatic fungi and their functional role in floodplain wetlands.

An initial survey of fungal diversity in a floodplain wetland (Chapter 3) clearly showed that fungi were present on a range of submerged and exposed substrates. A mesocosm experiment then examined the influence of leaf age and moisture availability on fungal dynamics (chapter 4). A period of terrestrial aging was found to promote fungal colonisation of Eucalyptus camaldulensis leaves during aquatic decomposition, potentially impact ing wetland food chains, water quality and carbon dynamics.

DNA from a range of fungal species was extracted from the sediments of a floodplain wetland undergoing drawdown (Chapter 5), while fungal biomass was below detection limits. The results suggest that fungi do not grow in wetland sediments. Rather, the sediments act as “seed bank” that stores fungal propagules until the wetland refills.

Aged E. camaldulensis leaves undergoing aquatic decomposition in river and wetland ecosystems were examined to compare fungal and Oomycete community dynamics (Chapter 6) and changes in leaf chemical composition (Chapter 7). Fungal biomass was consistently lower in wetlands, when compared to streams and Oomycete biomass comprised a substantial proportion of total fungal biomass at various times in both ecosystems. Initial increases in fungal and Oomycete biomass were correlated with temporary improvement in leaf food value. Infra-red mapping of these partially decomposed E. camaldulensis leaves (Chapter 7) showed changes in the distribution of polysaccharides, protein and lignin over time, and changes in lignin distribution occurred earlier in the wetland than in the stream. The activity of oxidative fungal enzymes was indicated as an important mechanism in the transformation of refractory carbon compounds in aquatic ecosystems.
Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Research procedures reported in the thesis carried out in the Ovens River Nature Conservation Reserve were undertaken under Department of Sustainability and Environment Permit No: 10004708 / File No:FF383072.

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Chapter 1

*General Introduction*

The role of micro-organisms in nutrient cycles has been widely recognised, and the integrated study of micro-organisms and the chemical changes they initiate in the environment has become known as biogeochemistry (Mitsch and Gosselink 1993a). In wetlands, studies of biogeochemistry have been focused primarily on the activities of anaerobic bacteria in the sediments (Boon and Sorrell 1991; Gutknecht, Goodman et al. 2006; Sorrell and Boon 1992b), and benthic and pelagic aerobic bacteria (Howitt, Baldwin et al. 2007; Wetzel 1992). Very little research exists that examines the role of aquatic fungi in the cycling of carbon in wetlands. Given the known importance of fungi in terrestrial systems, we might intuitively expect fungi to have some role in carbon cycling in wetlands, but this role had not been explored, much less quantified. As carbon dynamics take on increasing importance in the light of the enhanced greenhouse effect and global climate change, an examination of this role is timely.

1.1 **Ecological Studies of Fungi in Australia**

The most important previous study of the ecology of aquatic fungi in Australia was the doctoral thesis by Ken Thomas (Thomas 1992), and the subsequent publications (Thomas, Chilvers et al. 1989; Thomas, Chilvers et al. 1992). However, this study dealt with fungal dynamics in an upland stream, an ecosystem quite different to floodplain wetlands. Other studies have investigated fungal dynamics in the litter layer of eucalypt forests (Cabral 1985; Eicker 1973; Macauley and Thrower 1966; Orsborne 1987), fungal endophytes (Bertoni and Cabral 1988; Bettucci and Saravay 1993) and the effect of pathogenic fungi on eucalypts (Abbott, Vanheurck et al. 1993). There are no published studies of fungal dynamics from Australian floodplain wetlands, and very few from floodplain wetlands in other countries (Baldy, Chauvet et al. 2002; Chergui and Pattee 1993; Pattee and Chergui 1995; Singer 1988).

1.2 **Floodplain Wetland Ecosystems**

In Australia’s Murray-Darling Basin, floodplain wetlands are important cultural sites. Both aboriginal people and those arriving over the last two centuries have utilized them
for water, food gathering, forestry, flood mitigation and biodiversity conservation (Lloyd, Atkins *et al.* 1992). These wetlands also have spiritual significance for many people.

Ecologically, floodplain wetlands are important for a variety of reasons. They influence water quality (Mulholland and Kuenzler 1979), are biodiversity and productivity hot spots (Hillman and Quinn 2002) and ground water fed wetlands may act as a drought refuge for a range of different species (Baldwin and Humphries 2001). In many ways, the value of floodplain wetlands in Australia lies in their heterogeneity, each of more than 7000 wetlands on the Murray River floodplain (Pressey 1990) harboring a unique community (Lloyd, Atkins *et al.* 1992). Fungi are likely to be important components of these ecosystems, even though their interactions with other organisms have not yet been investigated.

1.3 Carbon Cycles in Wetlands

The concentration of carbon dioxide in the atmosphere has increased by one third in the last 150 years, while methane concentrations have increased by 140% (Reddy and DeLaune 2008). Wetlands are one of the major non-anthropogenic sources of these emissions, with natural wetlands producing methane in the range of 100-300 x 10^{12} grams of carbon per year (Chynoweth 1996; Reddy and DeLaune 2008). Carbon emissions from wetlands tend to increase with ecosystem productivity (Whiting and Chanton 1993), and floodplain wetlands can be highly productive systems (Briggs and Maher 1985). Wetlands are also known to store more carbon per hectare than most other ecosystem types (Houghton 1995; Schlesinger and Melack 1981; Whittaker and Likens 1973b). It is important that we understand what contribution aquatic fungi might make to these emissions, and how their presence might influence emission from other sources. Such an understanding would contribute to the design of wetland management practices that reduce carbon emissions and increase carbon storage.

1.4 Aim

The aim of this thesis was to examine the role of fungi in the decomposition of various carbon sources in the floodplain wetlands of Australian lowland rivers.

1.5 Approach

For this thesis it was deemed appropriate to take an exploratory approach, and to examine the fungal community of floodplain wetlands as a whole, rather than identify
fungal species present. So while it is acknowledged that identifying fungal taxa is important to determine the impacts of finer scale interactions within an ecosystem, only broad taxonomic and functional groups of fungi have been discussed in this project, and no attempt has been made to definitively identify fungal species.

### 1.6 Organisation of the Thesis

The scientific literature discussing carbon cycling in wetlands and, within this context, the activities of fungal decomposers are reviewed (Chapter 2). This review suggests that it is reasonable to assume that, of the total fungal community within wetlands, the saprobic fungi are most likely to have the strongest influence on wetland carbon cycles. However, as no study has investigated fungal dynamics in lowland river floodplain wetlands, it is important to first confirm that fungi are present, and which substrates they are colonising.

A pilot study was devised where fungal DNA was extracted from a variety of substrates within a floodplain wetland (Chapter 3). These substrates included wetland sediments under various moisture conditions, sediments from the nearby river, wetland macrophyte litter, and the abscised leaves of *Eucalyptus camaldulensis* (the river red gum) and *Salix alba* (white willow). These data indicated differences in fungal community structure between sediments and plant detritus. Changes were also observed with different sediment moisture contents, between sites in the wetland, and between sediments in the river and wetland. The fungal community structure on eucalypt leaves differed from that on other plant detritus.

Given these preliminary findings, a mesocosm experiment was designed to investigate fungal dynamics on *E. camaldulensis* leaves under different moisture regimes that may exist within the filling and drawdown cycle of floodplain wetlands (Chapter 4). As the seasonality of this cycle influenced the quality of the eucalypt leaf as a fungal substrate, both fresh leaves and leaves that had aged under terrestrial conditions on the floodplain were used. This experiment showed that fungal dynamics differed markedly between fresh and aged eucalypt leaves, demonstrating that leaching (either by submergence or aging on the floodplain), was an important process that removes leaf components that inhibit fungal colonisation. Importantly, a significant increase in fungal biomass that occurred on aged leaves immediately after being submerged was absent on fresh leaves.
After 60 days, the fungal community structure on fresh and aged leaves was similar, but biomass was significantly lower on fresh leaves. For all mesocosm treatments, fungal biomass was approximately ten times less than that reported from leaf litter in streams. Importantly, much of the fungi observed growing on leaves in the mesocosms was not true fungi but belonged to the Oomycetes (see Frontispiece). The biomass of these organisms is not assessed when the ergosterol method is used to estimate fungal biomass.

The presence of fungi on natural substrates was then examined in a field setting. In Chapter 5 the biomass and community structure of fungi in wetland sediments is explored, while in Chapter 6 fungal and Oomycetes biomass and community structure are examined on submerged floodplain aged leaves, and compared between river and wetland environments. Sections of leaves that had been “conditioned” in the river and wetland were then examined using Fourier transform infrared micro-spectroscopy (Chapter 7). Here the chemical changes occurring in the leaf substrate as fungal decomposition proceeded were examined over a time series. As these results are spatially explicit, the sequence in which various leaf components were degraded was revealed, and differences were observed between leaves incubated in the river and the wetland.

Finally, the experimental results are discussed in the context of other research into aquatic fungi in freshwater ecosystems (Chapter 8). While there is a growing body of knowledge regarding fungal dynamics on plant detritus in river ecosystems, our understanding of aquatic fungi in wetland and floodplain ecosystems is less complete. The areas of study important for a more complete understanding of the role of aquatic fungi in wetland carbon cycles are discussed, and research approaches are suggested.
2.1 Introduction

Carbon is life. Every carbon atom on planet Earth spends eternity passing into and out of living organisms. This is the essence of the carbon cycle, and the food chains and decomposition pathways that drive it. The rate at which carbon moves along these pathways has changed as a result of human activities including river regulation, flood mitigation and wetland drainage. Wetlands are highly productive systems capable of storing large amounts of carbon (Brinson, Lugo et al. 1981; Houghton 1995; Mitsch and Gosselink 2000; Whittaker and Likens 1973a), so processes that accelerate the transformation of stored wetland carbon into gaseous forms have the potential to contribute substantially to global climate change (Chynoweth 1996; Reddy and DeLaune 2008; Whiting and Chanton 1993). For example, over 10% of global carbon dioxide emissions each year result from the degradation and destruction of peatlands (Parish, Sirin et al. 2008). Thus, it is important that we understand the mechanisms of carbon transformations in wetlands and adjacent systems.

The floodplain wetlands of lowland rivers in Australia are often ephemeral, experiencing both terrestrial and aquatic conditions over time. While they are commonly highly productive in terms of aquatic vegetation, they also receive large amounts of plant litter from outside of the water body (allochthonous litter). As much of this litter is not directly grazed upon by fauna (Brinson, Lugo et al. 1981; Newman 1991), decomposition processes will determine how much carbon is stored in a wetland, and how much is released into the atmosphere. It is therefore essential to fully understand aquatic decomposition processes in these systems.

Floodplain wetlands are found adjacent to streams that, at least occasionally, flood beyond their banks and have an associated floodplain (Mitsch and Gosselink 1993b). These systems are generally characterized by a cycle of flooding and drying that entails alternation between terrestrial and aquatic conditions (Mitchell 1992). In Australia, examples of floodplain wetlands include ephemeral and permanent ox-bow lakes, and river red gum (Eucalyptus camaldulensis, Dehn.) forest wetlands. These water bodies
are commonly referred to as “billabongs” and the terms floodplain wetland and billabong are used interchangeably.

Australia’s floodplain wetlands differ from lakes in two important ways. Firstly, lakes generally attain a depth that allows thermal and chemical stratification to occur. This results in anoxic bottom layers and the dominance of anaerobic decomposition processes within the sediments. The relatively shallow depth of wetlands enables oxygenation of the water column and the surface layer of sediment, at least during winter. This means that 70-80% of decomposition is carried out by aerobic organisms (Kjoller and Struwe 1980). Secondly, carbon inputs to lake sediments are generally dominated by algae, which contain no lignin, whereas in wetlands carbon inputs are usually dominated by macrophytes, which are 20-30% lignin. Lignin is degraded slowly under anoxic conditions. Once lignified plant detritus becomes entrained within anoxic sediments it may be stored there for extensive periods (Westermann 1993).

The distinctive features of floodplain wetlands are that they are linear in form (often being former stream channels), both energy and matter pass through them in large quantities, and they maintain lateral and longitudinal connectivity to other ecosystems (Mitsch and Gosselink 1993b). Flood events are the main mechanism through which lateral connectivity and energy flow are achieved. These events provide a pulse of nutrients and organic matter, while also altering pH, salinity and dissolved oxygen, and influencing primary production. The alternation of flooded and dry conditions sets up temporal gradients in oxygen, nutrient and carbon contents of soils and sediments (Hart and McGregor 1980) that can affect the rate of microbial decomposition processes (Mitsch and Gosselink 1993a).

The floodplain wetlands have important ecological functions in Australian systems. They are able to improve the quality of water that passes through them, are biodiversity and productivity hot spots (Kingsford, Curtin et al. 1999), and act as a seasonal drought refuge for water birds (Kingsford and Norman 2002) and fish (Arthington, Balcombe et al. 2005; Magoulick and Kobza 2003). They are also important fish nurseries and waterfowl habitat due to rich phytoplankton and zooplankton communities that provide a foundation for the food chain. Extensive macrophyte growth may provide shelter and a refuge from predators.
However, each wetland is unique in terms of its physical and chemical characteristics and may support a distinctive community (Lloyd, Atkins et al. 1992). There are more than 7000 open water wetlands on the floodplain of the Murray River alone (Pressey 1986), covering 2200 km², an area equal to 17% of the floodplain (Margules and Partners, P and J Smith Ecological Consultants et al. 1990). These systems could therefore be considered important based solely on their extensive area.

2.2 The Breakdown and Decomposition of Plant Detritus

Much of the literature that describes the processing of dead organic material refers to both breakdown and decomposition. Here these terms are defined in the same manner as Boon (2006): breakdown is the gradual loss of mass from organic material by means of physical fragmentation, leaching and the conversion of organic carbon to inorganic forms, while decomposition (and mineralisation) describes the conversion of organic substances into carbon dioxide and methane (Boon 2006; Boulton and Boon 1991). In wetlands, decomposition is mediated by a variety of micro-organisms, while breakdown results from physical, chemical and biological processes. This review will focus primarily on decomposition in wetlands, and discuss the contribution of these decomposition processes to the overall breakdown of organic matter.

Further to this, the term “oxic” will be used to describe conditions where oxygen is available, while the term “aerobic” will be used to denote metabolic processes that require oxygen. Similarly, “anoxic” describes the absence of oxygen, and “anaerobic” denotes processes that do not require oxygen.

2.2.1 Aquatic plant litter decomposition

The breakdown and decomposition of organic plant material in aquatic systems is described as a three stage process (Bunn 1988a). It consists of:

(a) Leaching of soluble substances;
(b) Colonisation by micro-organisms and the commencement of decomposition through the activities of extra-cellular enzymes (microbial conditioning); and
(c) Physical fragmentation, mediated by mechanical forces and the activities of macro- (>2mm) and meio-fauna (<2mm) (Pidgeon and Cairns 1981; Webster and Benfield 1986).
Leaching is responsible for the largest mass loss from submerged litter in the initial stages of decay (Day 1983). In aquatic ecosystems the rate of leaching is not limited by precipitation, but is controlled by species specific litter quality and plant tissue type (wood, bark, leaves or other structures)(Baldwin 1999; O'Connell, Baldwin et al. 2000). Water soluble organic substances such as sugars, amino acids and organic acids, begin to leach from plant detritus immediately after immersion. Up to 30% of the organic carbon can be lost from litter after just 4 days in the water (Brinson 1977). The leaves of *E. camaldulensis* become leached within 72 hours (Glazebrook 1995), while leaching of soluble substances from woody debris can take more than a year (Thomas, Chilvers et al. 1992). The amount of DOC released from submerged twigs by leaching in the first 48 hours is proportional to the mass of wood immersed (O'Connell 1998), but for large woody debris, decay is confined to thin surface layers (O'Connor 1992). Relatively small amounts of organic substances are leached from particulate organic matter (POM) (O'Connell, Baldwin et al. 2000). Carbon that is leached from submerged litter into the water column is known as dissolved organic carbon (DOC).

Following leaching the litter is composed of refractory organic compounds. These compounds are largely insoluble plant structural polymers including cellulose, hemicellulose and lignin (Chamier 1985; Esau 1977). The leaves are colonised by microorganisms and decomposed at various rates, depending on the physical and chemical characteristics of the water body (Westermann 1993). The microbial community composition also changes over time as the substrate composition changes, utilising soluble carbon, cellulose and lignin sequentially (Moorhead and Sinsabaugh 2006). This process is known as “leaf conditioning”. Together, leaching and leaf conditioning may be responsible for up to 70% of the mass loss from submerged plant detritus (Findlay, Howe et al. 1990).

The decomposition rate is, by definition, the amount of carbon consumed from a resource over time. Carbon consumption by micro-organisms is influenced by factors such as moisture availability, temperature, the quality of the resource and the availability of oxygen (Baldwin 1999; Mackensen, Bauhus et al. 2003). While temperature, moisture and resource quality influence the metabolic rates of the microbial community, the availability of oxygen influences decomposition rates chiefly via changes to the microbial community composition. Under oxic conditions, submerged leaves are
colonised by communities dominated by aerobic fungi, while under anoxic conditions, anaerobic bacteria are dominant (O'Connell 1998). As anaerobic decomposition tends to proceed more slowly (Boon 2006; McLatchy and Reddy 1998; Webster and Benfield 1986), anoxia slows decomposition rates.

In aquatic systems, leaf conditioning improves the food value of the leaves for invertebrate consumers (Bärlocher 1982; Bärlocher 1985; Bärlocher and Corkum 2003). During conditioning, concentrations of nitrogen and phosphorus increase as structural polysaccharides are metabolised and microbial biomass accumulates (Suberkropp 1992; Suberkropp, Godshalk et al. 1976). In streams, fungi comprise the majority of the microbial biomass in the period prior to the point where leaves have maximum food quality (Findlay and Arsuffi 1989).

Fungi improve the food value of leaf detritus by adding fungal biomass (Bärlocher 1985; Bärlocher and Kendrick 1975), and enzymatically converting plant polymers into more digestible forms (Suberkropp 1992; Zimmer, Oliveira et al. 2005). While it has been suggested that these processes improve palatability to aquatic invertebrates (Kaushik and Hynes 1971), Suberkropp et al. (1983) found no relationship between fungal biomass and palatability. However, palatability is not the same as food value. Insects require sterols and long chain linoleic (ω3) polyunsaturated fatty acids for metamorphosis and pupation. These compounds are not available from plants or bacteria but can be provided by fungal tissues (Boulton and Boon 1991). Furthermore, fungi may provide nutrients to invertebrates that are critical for growth, maturation and reproduction (Albariño, Villanueva et al. 2008; Cargill, Cummins et al. 1985).

In aquatic systems, conditioned litter is consumed by a group of aquatic invertebrates functionally defined as “Shredders” (Merritt and Cummins 1978). These are mainly the larvae of invertebrates that shred and chew coarse particulate organic matter (CPOM), such as plant detritus. They include stoneflies, caddisflies, crane flies and midges (Cargill, Cummins et al. 1985; Reid, Quinn et al. 2008; Suberkropp, Arsuffi et al. 1983). This group of organisms is known to affect litter breakdown and decomposition rates in streams by digestion and respiration of the organic carbon (Golladay and Sinsabaugh 1991; Pidgeon and Cairns 1981), and mechanical fragmentation (Van Wensem, Van Straalen et al. 1997). However, invertebrate communities can differ markedly between
streams and wetlands (Sheldon, Boulton et al. 2002; Suter and Hawking 2002). While invertebrate communities in wetlands also rely on allochthonous carbon (Batzer and Wissinger 1996), shredders that are specifically adapted to process plant litter are rare in wetlands (Balcombe, Closs et al. 2007; Mulholland 1981). The influence of shredders on leaf breakdown can not be assumed to be the same for rivers and wetlands.

In a forested floodplain wetland on an Australian lowland river, fragmentation dominated the latter half of aquatic leaf break down and decomposition (Glazebrook 1995), a process known to take about 4 months (Briggs and Maher 1983). While fragmentation was the most important factor affecting leaf mass loss in streams (Golladay and Sinsabaugh 1991), a study in the littoral zone of a lake has suggested that fragmentation and microbial processes are of equal importance in non-flowing environments (Kominkova, Kuehn et al. 2000). Similarly, Janssen and Walker (1999) concluded that physical fragmentation and the proportion of carbohydrates were of equal importance in the control of leaf break down rates in a floodplain wetland on an Australian lowland river. As structural carbohydrates are the main energy source for microbes in submerged leaves, these studies suggest that invertebrate feeding and microbial activity gain equal importance in determining the rate of leaf breakdown in the absence of flow.

In aquatic ecosystems abiotic carbon loss, carbon mineralisation and mechanical fragmentation are important factors contributing to the overall rate of breakdown and decomposition of submerged leaf litter. Carbon loss is largely due to leaching, which is rapid and immediate. The composition of the decomposer community responsible for carbon mineralisation is heavily dependent on environmental factors such as dissolved oxygen, and varies between water bodies. Invertebrate activity, in combination with abiotic factors including flow, dominates litter breakdown rates, but microbial activity may be of equal importance to breakdown rates in the absence of flow.

### 2.3 The Influence of Environmental Conditions

Floodplain wetlands are highly dependent upon seasonal flooding. In a survey of 58 wetlands on the Murray River floodplain, Roberts (2006) reported that 86% were categorised as “lentic channel forms”. This wetland type includes ox-bow lakes and meander cut-offs, and former channels of various lengths in low-lying areas of the floodplain that are connected, or potentially connected, to the river at or above minimum
regulated flow (Pressey 1986). This wetland type accounted for 50% of all 6433 wetlands identified by Pressey in 1986. Other wetlands which are in higher areas of the floodplain receive water when river flows are above maximum regulated flow (Pressey 1986), and may dry out intermittently. However, minimum regulated flows have changed in recent years, and many wetlands in low-lying areas may now also dry periodically. Roberts (2006) reported that only 81% of lentic channel forms were conneted at minimum regulated flow.

The physical and chemical characteristics of a floodplain wetland can have a strong influence on the rate of plant litter decomposition, particularly in terms of the biological processes involved. In floodplain wetlands, one of the strongest influences on plant litter decomposition is the flooding and drawdown cycle, which is accompanied by seasonal temperature changes. These processes affect the decomposer community (invertebrates (Balcombe, Closs et al. 2007), fungi and bacteria) by influencing the availability of organic substrates (food), the availability of suitable habitat, and opportunities for reproduction. The influence of the water regime and seasonal changes is reflected in the measurable physical parameters. These include the hydrological regime, temperature, turbidity and sedimentation, salinity, pH, oxygen availability and redox potential, nutrients and litter quality. These factors have been shown to strongly influence decomposition rates and pathways (McLatchy and Reddy 1998).

2.3.1 The hydrological regime
Annual flooding supplies floodplain wetlands with carbon in the form of leaf litter, large woody debris (LWD) and leachates from the soil. Floodwaters also deliver nutrients including nitrogen and phosphorus (Baldwin and Mitchell 2000; Robertson, Bunn et al. 1999). Flood events flush and refill floodplain wetlands, creating the potential for high levels of primary productivity (Bayley 1991; Junk, Bayley et al. 1989). A significant amount of dissolved organic carbon (DOC) may also be returned to the river channel during a flood event from reservoirs on the floodplain such as wetlands (Hillman 1995; Sedell and Dahm 1990). While billabongs and other lentic water bodies on floodplains generally receive water rapidly during flood events, water is lost gradually by evaporation. Thus, while water quality may be similar to that of the river when the wetland is full, water quality parameters begin to vary from the river as soon as the connection is lost (Hillman 1986).
The onset of flood in a floodplain wetland brings a number of changes to the physical environment. These include soil/sediment anoxia, the importation and exportation of organic matter, nutrients and sediment, and shifts in pH and oxidation states (Baldwin and Mitchell 2000; Boon and Mitchell 1995; Mitsch and Gosselink 1993a). However, periodic flooding alternating with dry periods can increase plant productivity, enhance soil nutrient cycles, re-oxygenate root zones and flush toxins and wastes from the system (Mitsch and Gosselink 1993a). Flooding may influence plant litter breakdown differently depending on its position in the wetland. Neckles and Neill (1994) found that flooding increased litter mass loss rates where litter was lying above the sediment, but slowed decomposition below the sediment surface. These changes will have ramifications for wetland decomposition.

The timing and/or season of flooding also have a significant impact on the activity of the decomposer community in Australian wetland sediments. Flooding of wetlands in summer, as opposed to autumn/winter, can cause rapid increases in methane emissions from the sediments (Boon, Mitchell et al. 1997) and accelerated decomposition of floodplain leaf litter (Glazebrook and Robertson 1999). River regulation has altered the timing and season of flooding in many Australian river systems. Under regulated conditions the majority of small and medium floods are captured by headwater dams in winter/spring, and minor to moderate flooding occurs in summer due to irrigation releases (Frazier and Page 2006). The change in the timing of these flooding events from winter/spring to summer coincides with higher water temperatures. This affects both the metabolic activity of micro-organisms and the ability of the water and soils to retain oxygen. As a consequence, there are changes in the types of decomposition processes that predominate, and in the rate at which they proceed. Changes in flood seasonality also affect the primary production in floodplain wetlands (Robertson, Bacon et al. 2001).

Decomposition is faster in wetlands that are flooded for at least part of the year than at sites that are never flooded (Neckles and Neill 1994). This is because flooding accelerates leaching and the microbial conditioning phases of decay (Baldwin 1999; Glazebrook 1995). When flooding alternates with drying, both aerobic and anaerobic decomposer communities are able to contribute to organic matter decomposition. However, overseas research indicates that alternating oxic and anoxic conditions slows
decomposition (Mitsch and Gosselink 1993a). The duration of wet and dry periods and
the depth of inundation are also likely to affect the decomposition of leaves in wetlands.
However, the impact of these factors has not been specifically addressed. Sediment
drying has also been reported to induce the formation of acidic conditions where sulfidic
sediments are present (McCarthy, Conallin et al. 2006), which is likely to impact all
wetland biota in profound ways, including the decomposer community.

2.3.2 Temperature
Temperature affects the decomposition of plant material in wetlands through its influence
on the activity of the microbial community. The metabolic rate of micro-organisms
approximately doubles with each 10°C rise in temperature, then begins to plateau before
the temperature reaches a critical value where the organisms’ proteins begin to denature .
This relationship has been described as the “Q10 factor” (Kirschbaum 1995; Kirschbaum
2006). Many authors have demonstrated faster rates of leaf breakdown under warmer
conditions (e. g. Coûteaux, Bottner et al. 1995; Glazebrook and Robertson 1999; Guo
and Sims 2001; Wood 1974) and others have found higher microbial biomass in warmer
weather (Goncalves, Graca et al. 2007; Scholz and Boon 1993a). Temperature changes
can also trigger reproductive events in aquatic micro-organisms (Thomas, Chilvers et al.
1989). High levels of DOC in combination with high temperatures are known to cause
“black water events”. A hypoxic black water event is a catastrophic decrease in dissolved
oxygen caused by a sharp increase in oxygen demand by bacteria in the water column
(Howitt, Baldwin et al. 2007). Serious ecological consequences, including fish kills, may
ensue.

Temperature was the main driver of coarse woody debris decomposition on the floor of
Australian eucalypt forests (Mackensen, Bauhus et al. 2003), explaining more than 30%
of the variability in its decomposition rate (Campbell, James et al. 1992b). This is in line
with findings that refractory forms of organic matter from soils and litter are more
sensitive to changes in temperature than labile organic matter (Conant, Drijber et al.
2008).

2.3.3 Turbidity and sedimentation
Relative to their associated stream, floodplain wetlands are generally more turbid and
more highly coloured (Hillman 1986). Turbidity may be further increased by the activity
of livestock and feral animals (Hart and McGregor 1980). In shallow lakes particles such
as sediments and algal cells may go through a cycle of sedimentation and re-suspension (Scheffer 1998). This continuous deposition of sediments in floodplain wetland environments results in the burial of organic detritus, such as leaf litter, after it settles on the sediment surface.

The continued deposition of sediments over leaf litter can alter the quality of the leaves as a food resource for both invertebrates and micro-organisms. Sedimentation can reduce the availability of oxygen and nutrients, and limit opportunities for reproduction and dispersal. Janssen and Walker (1999) found that burial of decomposing leaves affected breakdown rates only where the leaves were considered to be fast decomposers. The breakdown of “slow” species was not inhibited by burial.

2.3.4 Salinity and pH

Where connectivity with the parent stream is relatively frequent, floodplain wetlands have water which is extremely fresh, varying from 50 to 2250 μS.cm\(^{-1}\) (Goonan, Beer et al. 1992; Hillman 1986). Salinity begins to influence the macro-biota at levels around 1000 μS.cm\(^{-1}\) (Goonan, Beer et al. 1992; Hart, Bailey et al. 1991; Nielsen, Brock et al. 2003). Where connectivity is less frequent, in terminal wetlands or where there are inflows of saline groundwater, high salinity (James, Hart et al. 2009) and hypersaline (Reid and Brooks 2000) conditions may occur.

Billabongs exhibit a wide range of pH values, and large changes can occur on a daily basis. This range is so large that it is certain to affect chemical equilibria within the water column and sediments, however, more than 80% of wetlands have mean pH values close to neutral (Hillman 1986). Rapid changes in pH have been recorded in association with decomposition, and short-lived increases in photosynthesis (Tan and Shiel 1993). Flooding may also lead to reduced oxidation states and shifts in pH that can reduce the availability of some nutrients (Mitsch and Gosselink 1993a).

The presence of sulfides in the sediments of floodplain wetlands provides the potential for the development of acid sulfate sediments. Where wetlands have been inundated for extended periods and where source water is of poor quality, salinity is high, soil pH is low or oxidised iron is visible on the sediment surface, oxidation of the sediments as a result of drying can result in the development of acid (Baldwin, Hall et al. 2007). This
has catastrophic effects on wetland biota (McCarthy, Conallin et al. 2006). It is believed that the ingress of saline groundwater inhibits activity by methanogenic bacteria and favours sulfate reducing bacteria. Where sulfate reducing bacteria consume the bulk of sediment carbon, hydrogen disulfide can build up in the sediments. Oxidation leads to the formation of sulfuric acid (Hall, Baldwin et al. 2006).

2.3.5 Oxygen availability and redox potential

Oxygen demand in wetlands can be as high as 2.7 gO$_2$.m$^{-2}$.day$^{-1}$ (Robertson, Healey et al. 1997), leading to low dissolved oxygen concentrations in the water column and sediments (Ford, Boon et al. 2002). This may limit aerobic decomposition in the sediments to a surface layer of less than 1cm thickness (Cappenberg 1988), or exclude it entirely. Iron oxide mediated photochemical degradation of organic carbon in the water column also contributes to oxygen demand (Howitt, Baldwin et al. 2004). As discussed earlier, high oxygen demand may accompany floods in floodplain forest wetlands, as the inundation of floodplain litter leads to sudden increases in DOC.

It is generally accepted that decomposition in wetlands occurs more slowly under anoxic conditions (Brinson, Lugo et al. 1981; Neckles and Neill 1994). In most cases, however, there are many factors involved in determining decomposition rates, and the importance of oxygen is variable. Godshalk and Wetzel (1978b) found slower breakdown of POM under anoxic conditions but concluded that the effect of dissolved oxygen was minor in comparison with temperature and the initial nitrogen concentration of the substrate. This was in contrast to findings that DOC decomposition was strongly influenced by oxygen concentrations (Godshalk and Wetzel 1978a). While anoxia slows the degradation of plant detritus on inundated floodplains, the rate of DOC uptake from the water column is unaffected by anoxic conditions (O'Connell 1998).

While fungi may be present in anoxic environments, there is no published work demonstrating that they are able to participate actively in decomposition under these conditions. Fungal growth is severely limited below 4% oxygen saturation in waterlogged soils and 2% saturation in decaying wood (Swift, Heal et al. 1979), and activity ceases with the onset of anoxia (Lopez-Real and Swift 1977). Research has shown that submerged E. camaldulensis leaves are colonised by aerobic fungi under oxic conditions, while under anoxic conditions they are colonised by anaerobic bacteria

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(O’Connell, Baldwin et al. 2000). Therefore, the alleviation of sediment anoxia by wetland drawdown and drying may lead to more complete decomposition of plant material in wetlands, while continuing sediment anoxia will restrict decomposition to anaerobic pathways.

2.3.6 Nutrient availability

Murray River billabongs are nutrient rich environments in terms of nitrogen and phosphorus (Goonan, Beer et al. 1992; Gribben 2000), and billabongs in other areas have also been shown to be nutrient rich (Hart and McGregor 1980; Leahy, Tibby et al. 2005). Nutrient enrichment accelerates leaf breakdown in streams (Suberkropp 1995; Suberkropp and Chauvet 1995; Webster and Benfield 1986), and higher rates of litter decomposition in a Murray River floodplain wetland have recently been correlated with higher nutrient concentrations (Watkins, Quinn et al. 2010). In streams, higher nutrient concentrations may result in decomposing leaves becoming nutrient enriched without observed changes in mass loss (Elwood, Newbold et al. 1981) and fungal activity, respiration and sporulation rates may also increase (Suberkropp 1991b). Microorganisms are also known to use solid substrates and DOC differently in waters of differing trophic status (Baldy, Gobert et al. 2007).

The decomposition of leaf material results in net losses of nitrogen, phosphorus, calcium, magnesium and potassium (Day 1983). Losses of nitrogen have been observed within 3 days of immersion (Glazebrook and Robertson 1999). Inundation of wetland sediments after a period of drying results in an initial flush of nitrogen from the sediments to the water column (Baldwin and Mitchell 2000), and additional nitrogen leached into the water from plant litter may lead to high concentrations of dissolved nitrogen.

The nitrogen content of the substrate can also influence decomposition rates (Gessner and Chauvet 1994). The leaves of sclerophyllous plants such as eucalypts have C:N ratios higher than 100:1 (Boon 2006; Glazebrook and Robertson 1999). When using these leaves as a substrate, large amounts of external nitrogen may be needed to sustain microbial metabolism (Boon 2006). Aquatic hyphomycete fungi are capable of utilizing dissolved inorganic nitrate and ammonium from the water column (Thornton 1963) to address this shortfall. Thus, submerged leaf litter colonised by aquatic fungi may become enriched with nitrogen (Glazebrook and Robertson 1999).
While bioavailable forms of phosphate are leached from leaves when they are submerged (Baldwin 1999), fungi colonising leaves are able to take up phosphate from the water column. This can result in observed relative increases in leaf phosphorus content as leaves undergo aquatic decomposition (Glazebrook and Robertson 1999). Leaf breakdown proceeds faster where phosphate concentrations are higher (Chauvet 1987; Meyer 1980; Rosset, Bärlocher et al. 1982; Suberkropp 1991b; Suberkropp, Godshalk et al. 1976), and this is likely to be due to a stimulating effect of phosphorus on microbial activity.

The re-wetting of wetland sediments can release a pulse of phosphorus into the water column (Baldwin and Mitchell 2000). While the origin of this phosphorus is likely to be plant litter (Qiu, McComb et al. 2003), increased moisture availability in dry sediments leads to increases in microbial biomass, with a corresponding increase in phosphorus derived from microbial sources (Qiu, McComb et al. 2003). Thus, the flux of phosphorus from wetlands sediments on rewetting is regulated by microbial activity.

Dissolved organic carbon can undergo photodegradation reactions. Leachate from *E. camaldulensis* leaves can be chemically degraded upon exposure to light, a reaction which results in CO$_2$ production and reduced concentrations of aromatic structures and double bonds (Howitt, Baldwin et al. 2004). Metal ions in solution can increase the rate at which naturally occurring DOC undergoes photolysis (Howitt, Baldwin et al. 2004; Zepp, Callaghan et al. 1998; Zepp, Faust et al. 1992). DOC photodegradation rates increase when iron oxide is present in the water column (Howitt, Baldwin et al. 2004). Humic and fulvic substances in solution are able to reduce Fe(III) to Fe(II), a reaction which also produces CO$_2$ (Miles and Brezonik 1981; Voelker, Morel et al. 1997). As freshly produced iron oxides are most reactive, photodegradation is especially relevant in wetlands and shallow lakes where anoxic layers in the water column may form in warmer weather. The mixing of oxic and anoxic waters is likely to generate fresh iron oxides, which may further increase oxygen consumption (Howitt, Baldwin et al. 2004). Under the anoxic conditions found in submerged sediments and reduced soils, fungi with metabolic requirements for manganese may also be present (Aguiar, de Souza-Cruz et al. 2006; Jennings and Lysek 1996; Kapich, Steffen et al. 2005; Keishi, Yassoo et al. 2006; Miyata, Tani et al. 2004; Thompson, Huber et al. 2005; Wainwright 1992).
2.3.7 Litter type / substrate quality

The quality of organic matter has a strong influence on decomposition rates, and on the decomposer community structure (Gulis 2001). Decomposition rate varies with the proportions of labile and refractory organic substances in plant litter. For example, decomposition rate has been found to vary with proportions of hemicellulose, cellulose and lignin in wetland macrophytes (Gessner and Chauvet 1994; Godshalk and Wetzel 1978b; Suberkropp, Godshalk et al. 1976; Webster and Benfield 1986) and in the leaves of riparian trees (Janssen and Walker 1999). High lignin content also confers slower decomposition, with twigs decomposing more slowly than leaves (Shearer and von Bodman 1983). The seasonality of litter fall may also be a critical factor in litter decomposition. This will affect the length of time litter is air dried on the floodplain before inundation, the temperature at inundation and whether leaves are still green when they are inundated (Boon 2006). Leaf age has been shown to strongly influence the speciation and bioavailability of organic matter leached from the leaves of *Eucalyptus camaldulensis* (Baldwin 1999).

Litter may be derived either from within the wetland (autochthonous carbon) or from sources outside the wetland, i.e. the floodplain (allochthonous carbon). Autochthonous carbon sources are largely derived from macrophytes and algae, while allochthonous carbon consists largely of the litter of riparian vegetation. Autochthonous carbon sources in Australian floodplain wetlands, such as *Phragmites* sp and *Typha* sp., have leaves which senesce in situ and begin the decomposition process in what is known as the “standing dead” position (Aseada, Nam et al. 2002; Gessner 2001; Kominkova, Kuehn et al. 2000). This group of plants forms a large proportion of the biomass produced in wetlands, and on the shores of lakes and rivers (Kuehn and Suberkropp 1998a; Pieczynska 1993). Fungi play a dominant role in the decomposition of standing dead leaf litter (Gessner 2001; Kuehn, Lemke et al. 2000; Kuehn and Suberkropp 1998a; Newell 2001; Newell, Moran et al. 1995; Van Ryckegem, Gessner et al. 2007). Rates of standing dead litter decomposition have been reported to be in the same order of magnitude as plant production rates, leading to minimal litter accumulation (Jackson, Foreman et al. 1995).

Algae are another significant autochthonous carbon source in wetlands. In contrast to macrophytes, algal production is often consumed directly by zooplankton and
invertebrates in wetlands (Boon 2000; Bunn and Boon 1993). During algal blooms, large amounts of algal biomass may precipitate to the sediment surface. These cells are quickly decomposed because they lack the structural carbohydrates that are characteristic of higher plants.

The leaves of *Eucalyptus* sp. comprise a large proportion of the allochthonous carbon reaching floodplain wetlands in Australia. In the aquatic phase they are colonised by both fungi and bacteria. The activity of fungal decomposers on the leaves of *E. globulus* has been compared with that on European leaf species (Bärlocher and Graça 2002; Canhoto and Graça 1996; Chauvet, Fabre *et al*. 1997). The main difference between these leaf types is the composition of the fungal community. While European species support an ‘autumn’ community, *E. globulus* leaves support ‘summer’ species, and these communities correspond with the season of leaf accession (Bärlocher and Graça 2002). A delay in colonisation relative to European leaf species (Canhoto and Graça 1996), and the use of the stomata as a point of access to leaf mesophyll have also been reported (Canhoto and Graça 1999).

### 2.3.8 Carbon compounds inhibiting biological processes

Plant tissues can also contain substances that are capable of inhibiting growth and activity in micro-organisms (Bärlocher and Oertli 1978). These include tannins, phenolic compounds and essential oils (Boon and Johnstone 1997). These substances may act synergistically to regulate microbial distribution and community structure. Antifungal compounds provide plants with resistance to fungal infection, especially in woody tissues (Cromack and Caldwell 1992; Mackensen, Bauhus *et al*. 2003). Leaching removes many of these inhibitory substances from dead plant material, and may enhance fungal colonisation (Bärlocher, Canhoto *et al*. 1995; Canhoto and Graça 1996; Dix and Webster 1995). Some fungi are able to neutralise these compounds with laccases and phenol oxidases in order to degrade lignin (Dorado, van Beek *et al*. 2001; Jönsson, Palmqvist *et al*. 1998; Leonowicz, Cho *et al*. 2001).

Tannins are a heterogeneous group of phenolic compounds defined by their ability to precipitate proteins and react with ferric chlorides (Boulton and Boon 1991). They are also able to complex with cellulose and hemicellulose (Zucker 1983), making them more resistant to decomposition (Boulton and Boon 1991). The tannin content of plant detritus
influences the rate of leaf processing due to fragmentation by invertebrates and microbial conditioning (Campbell and Fuchshuber 1995; Canhoto and Graça 1995; Gessner and Chauvet 1994; Suberkropp, Godshalk et al. 1976).

Tannins may be either hydrolysable or condensed. While both types have inhibiting effects on fungal colonisation, they differ in the mechanism of inhibition. Condensed tannins are able to inhibit both enzyme activity and growth, while hydrolysable tannins inhibit growth only (Nichols-Orians 1991). Their inhibitory effects can be attenuated in the presence of Al$^{3+}$, Fe$^{3+}$ or Mn$^{2+}$ ions (Marschner and Kalbitz 2002). However, Juntheikki and Julkunen-Tiitto (2000) found that while condensed tannins are able to bind with hydrolytic enzymes and precipitate them, enzyme activity may remain unaffected.

The role of polyphenols as defensive compounds against fungi in aquatic environments is generally accepted (Bärlocher, Canhoto et al. 1995; Bunn 1988b; Canhoto and Graça 1999). Leaching of soluble phenols from leaves can result in faster microbial colonisation (Bunn 1988b). The polyphenolics in eucalypt leaves have a deterrent effect on invertebrate shredders and micro-organisms (Canhoto and Graça 1995; Canhoto and Graça 1999; Graça, Pozo et al. 2002). This is thought to be due to their ability to bind fungal and invertebrate digestive enzymes and block digestion (Graça, Pozo et al. 2002). Fungal sensitivity to phenols seems to be species specific (Canhoto and Graça 1999), partly explaining why distinct fungal communities develop on eucalyptus leaves, as opposed to deciduous European species (Alnus glutinosa, Castanea sativa and Quercus faginea)(Canhoto and Graça 1996).

Terrestrial, wetland and littoral plants have been found to be a major source of polyphenols in temporary wetlands (Serrano 1992). While large influxes of polyphenols to wetlands due to leaching from terrestrial litter have been correlated with rainfall events (Serrano 1992), polyphenols are also a by-product of the decomposition of lignin (Kirk and Farrell 1987) and tannins (Serrano 1992). Oxidative breakdown of lignin by fungal enzymes produces quinones (6-membered rings with 2 carbonyl groups), which are able to complex with proteins, polypeptides, amino acids and amino sugars via the amino group (Suberkropp, Godshalk et al. 1976). This complexation provides resistance to decomposition (Dix and Webster 1995).
Leaf essential oils are used by the plant as a defence against the activities of microorganisms and herbivores (Stone and Bacon 1994; Terzi, Morcia et al. 2007). Eucalypt essential oils may depress or completely inhibit the growth of selected aquatic hyphomycetes, even more effectively than polyphenols (Canhoto and Graça 1999; Graça, Pozo et al. 2002). This is probably due to their interference with fungal enzymes (Canhoto, Bärlocher et al. 2002). In Australian systems, high concentrations of essential oils have also been shown to inhibit the decomposition of leaf litter (Bailey, Watkins et al. 2003; Boon and Johnstone 1997). The anti-fungal ingredient of eucalyptus oils is 1,8-cineole (Terzi, Morcia et al. 2007). The leaves of Eucalyptus camaldulensis have a 1,8-cineole content in the range of 12.7 to 71.7% of total terpenoid yield (the overall mean terpenoid yield was 1.86%, w/w, leaf dry wt). Leaves with the higher proportion of 1,8-cineole were found to be more resistant to attack by grazing insects (Stone and Bacon 1994).

The oils within Eucalyptus leaves can remain intact well after senescence and abscission because they remain locked within oil vesicles. These vesicles become leaky over time, so these oils can continue to inhibit microbes and invertebrates for some time after immersion (Canhoto, Bärlocher et al. 2002). Chironomid larvae have been observed to recognise and avoid oil vesicles, while Tipula larvae showed no indication of similar abilities (Canhoto and Graça 1999).

2.4 The Wetland Biota

Biodiversity in floodplain wetlands (billabongs) may exceed that found in the adjacent channel (Bornette, Amoros et al. 1998). High densities of pelagic and epiphytic algae are common, and the phytoplankton can be diverse (Gell, Sluiter et al. 2002; Reid and Ogden 2009; Shiel 1983). Similarly, these water bodies carry distinctive and diverse zooplankton communities (Hillman 1986; Nielsen, Hillman et al. 2002; Ning, Nielsen et al. 2008; Tan and Shiel 1993). The billabong environment favours macrophyte growth, and the banks are usually well vegetated and lightly grazed when compared with stream banks (Hillman 1986). Macrophyte communities exhibit considerable diversity, and vary widely between wetlands (Brock 1994; Reid and Quinn 2004; Robertson, Bacon et al. 2001).
Wetland macro-invertebrate communities differ substantially from those found in streams (Balcombe, Closs et al. 2007; Boulton and Lloyd 1991; Hillman and Quinn 2002), and often display high diversity (Goonan, Beer et al. 1992). Communities vary between wetlands, e.g. between billabongs with different hydrological regimes (Quinn, Hillman et al. 2000; Sheldon, Boulton et al. 2002), but are similar where wetland characteristics are similar (Marchant 1982).

Wetland fish communities tend to be dominated by the European carp (Cyprinus carpio) other introduced species such as Redfin (Perca fluviatilis), Tench (Tinca tinca) and Mosquito fish (Gambusia affinis) and small native species such as Western Carp Gudgeon (Hypseolotris kluinzeri) and Flathead Gudgeon (Philypnodon grandiceps) (Ellis and Meredith 2004; Hillman 1986; McNeil and Closs 2007).

Billabongs and other floodplain wetlands are also home to waterfowl in large numbers and considerable diversity (Kingsford and Norman 2002), frogs (Healey, Thompson et al. 1997), reptiles (e.g. red bellied black snake (Pseudechis porphyriacus) (Shine 1987) and long necked tortoise (Chelodina longicollis) (Kennett, Roe et al. 2009)), platypus (Ornithorhynchus anatinus) and water rats (Hydromys chrysogaster) (Grant and Temple-Smith 1998; Hillman 1986; Reid and Brooks 2000; Woollard, Vestjens et al. 1978).

2.4.1 River red gums

In temperate regions of eastern Australia, the dominant tree species on the margins of floodplain wetlands is the river red gum (Eucalyptus camaldulensis). River red gums generally occur as woodlands (Bacon, Stone et al. 1993), but may form dense forests in flood prone areas adjacent to rivers. Thus, the plant litter on floodplains in these regions is often dominated by the leaves of this species (Glazebrook and Robertson 1999; Robertson, Bunn et al. 1999). In addition, wood production from floodplain forests can yield up to 900 gC.m$^{-2}$.yr$^{-1}$ (Briggs and Maher 1983).

$E.\ camaldulensis$ litter, composed of leaves, twigs, bark and fruit, often in large quantities (Glazebrook 1995), is one of the major sources of DOC in Australian lowland rivers (O'Connell, Baldwin et al. 2000). This litter can account for up to 290 gC.m$^{-2}$.yr$^{-1}$ (Briggs and Maher 1983; Robertson, Bunn et al. 1999). The wood of this species decomposes slowly (O'Connor 1992), with an estimated life on the floodplain of 375
years (Mackensen, Bauhus et al. 2003). This creates a large pool of woody debris on the floodplain and within aquatic environments (MacNally, Parkinson et al. 2002).

Physico-chemically, wood differs substantially from leaves. Wood has a higher carbon to nitrogen ratio, higher density, lower concentrations of pectins, lower surface to volume ratio and lower parenchyma to sclerenchyma ratio (Erickson, Blanchette et al. 1990c). As a carbon resource, submerged wood is variable in size, while leaves have more or less uniform size. The input of wood to the aquatic environment is episodic, while leaf inputs are seasonal. Maximum litter inputs occur in summer, although leaf accession occurs throughout the year in lower amounts (Glazebrook and Robertson 1999). Woody substrates also have a much longer residence time in aquatic habitats (Shearer 1992). Thus, wood and leaves are vastly different substrates for the aquatic decomposer community.

In terrestrial and aquatic environments wood pieces decay from the outer layer inwards, and decay rates increase exponentially towards centre layers as the wood begins to crack and split (O'Connor 1992). However, overall decay is relatively slow, and woody debris is consistently present on the floodplain over time. Thus, wood serves as a reservoir of microbial propagules. For example, the period required for twig break down can exceed 390 days (Thomas 1992). Over this period, newly available resources can be quickly colonised from pre-existing populations, and seasonal changes in resource availability have little influence on the establishment of new populations.

Leaves represent a more transient resource than wood. E. camaldulensis leaves take 2-4 months to break down in the aquatic phase, with a half life of 80 days (Briggs and Maher 1983). The leaves decay according to an exponential decay pattern at rates similar to other eucalypt and temperate hardwood species (Briggs and Maher 1983). Results comparing decay rates in lentic and lotic systems have been varied. Glazebrook (1995) observed slower break down in lentic sites of a forested wetland. Contrary to this, Janssen and Walker (1999) found that the break down rate of river red gum litter did not differ between stream and wetland sites. These results may be influenced by season, which corresponds with differences in dissolved oxygen, temperature and moisture availability. As rate of decay increases with temperature, maximum E. camaldulensis leaf inputs generally coincide with maximum temperatures, and therefore maximum decay
rates. However, higher temperatures may also limit dissolved oxygen concentrations and lead to wetland drying. Litter inputs may also increase if eucalypt trees experience moisture stress (Stone and Bacon 1994; Stone and Bacon 1995).

2.5 Wetland Carbon Cycles

On a global scale, wetlands are important in the biogeochemical cycling of carbon (Westermann 1993). Wetlands can be extremely productive systems but grazing of this primary production is limited because dead plant material is comprised primarily of complex plant polysaccharides and lignins (Benner, Maccubbin et al. 1984; Gessner, Schieferstein et al. 1996; Wetzel 1990; Wetzel and Howe 1999) and is not easily digested. This means that much of the carbon incorporated into plant biomass is not consumed by higher trophic levels, but passes into the detrital food web (McLatchy and Reddy 1998). For example, *Phragmites australis* (Cav.) Trin. Ex Streud. is high in ligno-cellulose and reed stands of this species may have net above ground production in excess of 1 kg dry mass per square metre of surface area (Gessner 2001). As a consequence most of the biomass from these plants is shed as litter. This litter enters the detrital pool where it is decomposed by fungi, bacteria and invertebrates (Jackson, Foreman et al. 1995; Wetzel 1990). The insoluble fraction of plant detritus that is not mineralised is stored as sediment organic carbon.

Wetland soils contain about one third of all of the organic matter that is stored in the world’s soils (Mitsch and Gosselink 1993a). If one accepts the Ramsar definition of wetlands (Convention on Wetlands (Ramsar)), 40% of global terrestrial carbon may be stored in wetlands. Peatlands and forested wetlands are particularly important as carbon sinks. Deforestation and agricultural use of these wetlands accelerates the degradation of the soil humus, causing significant flux of greenhouse gases to the atmosphere (Aiken, McKnight et al. 1985).

The most common conceptualisation of the wetland food web is that of the “grazing food chain”, where phytoplankton are the primary producers that are consumed by zooplankton, which are then consumed by fish (Elton 1927). While this is occurring, however, the metabolic activities of micro-organisms are utilising much of the available carbon in the wetland. These resources are in the form of living and dead phytoplankton and plants and non-living carbon compounds. As these resources are mineralised gaseous
carbon compounds are produced, and released into the atmosphere. Residual carbon, that carbon that is not respired or incorporated into biomass, is stored in the sediments and water column. This carbon is referred to as humic and fulvic substances. Residual biomass in plant detritus, and the biomass of decomposer organisms, is now known to be as important as primary production as a basal resource in aquatic food webs (Hessen 1998; Pace, Cole et al. 2004; Reid, Quinn et al. 2008).

Biomass undergoes both physical and chemical changes as it undergoes decomposition. It is partitioned by particle size into POC and DOC. POC may be further classified as either fine (FPOC) or coarse (CPOC) (e.g. leaf litter and woody debris). Decomposition of DOC and FPOC in floodplain wetlands is largely mediated by bacteria (Baldwin 1999; Boon 2006), while fungi dominate the aquatic decomposition of CPOC (Suberkropp 1991a; Suberkropp and Klug 1976) under oxic conditions (O’Connell 1998).

The fluxes and storages in a typical wetland carbon cycle are illustrated in Figure 2.1, which is based on a diagram published by Webster and Benfield (1986). It shows how inorganic carbon from the atmosphere is incorporated into biomass via photosynthesis. This plant biomass also produces carbon dioxide and acts as a conduit for methane to pass from the sediments into the atmosphere. As this plant biomass senesces and dies, carbon is leached into the water column. Carbon is also leached from allochthonous litter and floodplain soils. The resulting DOC is mineralised by aerobic or anaerobic bacteria in the water column, depending on the availability of dissolved oxygen. Carbon dioxide respired by these bacteria is either dissolved, escapes to the atmosphere, or is converted into other inorganic carbon ions, depending on the pH of the water. Insoluble carbon compounds from plant, algal and animal detritus are mineralised by fungi and bacteria under submerged conditions. Residual carbon passes into the surface sediments, thence to anoxic sediments below the surface where carbon mineralisation is dominated by aerobic and anaerobic bacteria, respectively. These processes are discussed in greater detail below.
2.5.1 Primary and secondary production

The major route via which carbon dioxide is transformed into organic carbon is photosynthesis (by both plants and algae). Aquatic macrophyte production in wetlands may be very high (Bott, Barnes et al. 1983; Briggs and Maher 1985; Roberts and Ganf 1986) and is often stimulated by wetland drying and re-wetting (Briggs and Maher 1985). Litter production by *E. camaldulensis* in Australian floodplain wetlands is in the order of 120 - 700 g dry weight.m\(^{-2}\).yr\(^{-1}\) (Briggs and Maher 1985; Campbell, James et al. 1992a). This represents a substantial contribution of carbon to the wetland systems and floodplain soils. The presence of aquatic macrophytes may also influence the flow of methane from the sediments to the atmosphere (Boon 2000; Boon and Mitchell 1995; Boon and Sorrell 1991; Sorrell and Boon 1992a).

The river red gum (*E. camaldulensis*) is the dominant tree species around many Australian floodplain wetlands. The total litterfall from the river red gum is similar to other eucalypt species (~400 g.m\(^{-2}\).yr\(^{-1}\)), except there is a lower proportion of leaves (~25%) (Briggs and Maher 1983). Reid et al. (2008) measured direct inputs of litter into
streams which had riparian vegetation dominated by *E. camaldulensis*. They found litter inputs were 550 – 617g AFDW.m\(^{-2}\).yr\(^{-1}\), with lateral litter movement accounting for <10% of total inputs. This species sheds more litter in dry periods, and this generally corresponds with periods when wetlands are dry. Standing stocks of river red gum litter on a reach of the Darling River, NSW, were found to be 2.7 kg/m\(^2\) for twigs, 1.5 kg/m\(^2\) for bark, 1.5 kg/m\(^2\) for leaves, and 0.8 kg/m\(^2\) for gum nuts (Francis and Sheldon 2002).

The productivity of decomposer micro-organisms themselves can also be considered as an important carbon sink (Buesing and Gessner 2006), although less persistent than plant carbon (Findlay, Howe *et al*. 1990). In a freshwater marsh in Switzerland, Buesing and Gessner (2006) found that the annual productivity of bacteria on submerged plant litter under aerobic conditions far outweighed fungal production. Annual productivity was 18.8 and 2.4 mg C.g\(^{-1}\) C for bacteria and fungi respectively. Together, bacteria and fungi accounted for ~1500 g C.m\(^{-2}\).yr\(^{-1}\), while plant productivity in the same wetland was ~600 g C.m\(^{-2}\).yr\(^{-1}\). This indicates that the microbial community are important producers in wetlands.

Food chains represent the dominant pathway for carbon transformation in many systems. In wetlands, trophic relationships are complex. Bunn and Boon (1993) used stable isotopes to investigate food webs in Australian floodplain wetlands (billabongs or oxbow lakes). Based on the premise that consumers are slightly (~1‰) \(^{13}\)C-enriched when compared to their food source, *Cherax sp.* (a freshwater crayfish) was identified as a major consumer of detrital carbon. However, they found that all the major consumer groups were \(^{13}\)C-depleted when compared to the riparian and macrophyte vegetation, implying that an unknown \(^{13}\)C-depleted carbon source was being consumed simultaneously with vegetation. Methanotrophic bacteria and planktonic Chlorophyta were suggested as possible sources of this carbon. While fungi tend to be \(^{13}\)C-enriched relative to their substrate, this varies with substrate (Mayor, Schuur *et al*. 2009), between species and with growth stage (Henn, Gleixner *et al*. 2002), and between respiration and fermentation metabolic pathways (Henn and Chapela 2000). This means that fungal decomposers remain as possible candidates for this unknown carbon source. Moreover, Reid *et al*. (2008) found that biofilms (comprised of fungi, bacteria and algae) on submerged leaves of *Eucalyptus* sp. were an important carbon source in lowland streams. Given that fungal tissues provide nutrients vital to the diets of aquatic invertebrates (see
section 2.2.1), aquatic saprobic fungi provide a conduit for carbon flow between submerged litter and wetland consumers.

2.5.2 Sources of DOC

Vegetation is an important source of dissolved carbon (DOC) in aquatic systems. Of the particulate ligno-cellulose derived from wetland plants, 10-40% is converted to DOC prior to being consumed by micro-organisms (Benner and Opsahl 2001). Up to 25% of the carbon in river red gum leaves is readily leachable upon inundation (Glazebrook and Robertson 1999). Francis and Sheldon (2002) calculated that red gum litter had the potential to release 235g/m² of DOC, 80% of which would be derived from the leaves. Large amounts of DOC are also leached from these leaves on the floodplain prior to entering the aquatic environment (Baldwin 1999). Both terrestrial and aquatic leaching result in losses of polyphenols and essential oils from leaves (Serrano and Boon 1991). These compounds inhibit enzymatic processes in invertebrates and micro-organisms (Bengtsson 1992; Canhoto and Graça 1995; Canhoto and Graça 1999; Serrano and Boon 1991). For this reason, floodplain aging may be an important factor influencing the rate of microbial colonisation of leaf litter once submerged.

In the Murray-Darling Basin, biomass and concentrations ofDOC in wetlands may be up to three orders of magnitude greater than in the river channel (Boon 1991; Briggs, Maher et al. 1993; Briggs and Maher 1985; Briggs, Maher et al. 1985; Hillman and Shiel 1991) and DOC has been found to be negatively correlated with water level (Briggs, Maher et al. 1993; Lloyd, Atkins et al. 1992), indicating evaporative concentration.

Laudon et al. (2004) showed that the export of DOC from boreal catchments was positively correlated with the proportion of each catchment designated as wetlands. In particular, the total area of wetlands had a strong effect on the seasonality of DOC exports. Results such as these suggest that the timing and extent of DOC imports into aquatic environments may be altered as a response to global climate change (Tranvik, van Hees et al. 2004).

In the stationary waters of wetlands pulsed litter inputs and the associated rapid leaching can result in profound changes in water quality. The most important of these changes is the rapid decrease in dissolved oxygen, as aerobic organisms in the water column
metabolise DOC and use up oxygen in the process. Leaf aging on the floodplain prior to flood events reduces their contribution to DOC with flood onset (Baldwin 1999). Permanent inundation of litter accelerates decomposition relative to permanently dry conditions (Langhans and Tockner 2006). However, cyclic flooding and drying leads to faster decay relative to permanent flooding in warmer climates (Battle and Golladay 2001; Ryder and Horwitz 1995), while no difference was found in cooler climates (Inkley, Wissinger et al. 2008). Accelerated decay in winter relative to summer has also been reported (Langhans and Tockner 2006). This suggests interplay between inundation, temperature and dissolved oxygen concentrations that ultimately dictates aquatic litter breakdown rates.

The concentration of DOC in unpolluted river water is commonly 1–5 mgC.L⁻¹, but may reach 10 mgC.L⁻¹. Concentrations of labile DOC in this range may be turned over twice a day by planktonic bacteria (Findlay and Sinsabaugh 1999). Previously, about 25% of DOC was considered biodegradable (Volk, Volk et al. 1997). However, O’Connell et al. (2000) found that 40% of the DOC released from river red gum leaves was used rapidly by planktonic bacteria and there is evidence that bacteria are also able to use higher molecular weight compounds including polyphenols and ligno-cellulose (Findlay and Sinsabaugh 1999). High extracellular enzyme activity in the water column of Australian billabongs has been attributed to the metabolic activity of bacteria metabolising DOC (Boon 1989; Boon 1990; Boon and Sorrell 1991; Scholz and Boon 1993a; Scholz and Boon 1993b).

In addition, litter and woody debris from riparian eucalypt forests and woodlands, which is slow to decompose (Hutson and Veitch 1985; Mackensen, Bauhus et al. 2003; Nagy and MaCauley 1982; Presland 1982), can build up on the floodplain and dry wetland sediments. These wetlands consequently hold significant reservoirs of carbon, in both living and non-living forms. During a flood event 25-30% of the leaf mass can be transformed into DOC (Glazebrook and Robertson 1999; O’Connell 1998).

2.5.3 **Utilisation of DOC in the water column**

Research conducted in marine environments has shown that DOC is utilised by bacteria. This has become known as the microbial loop (Azam and Cho 1987; Meyer 1994; Pomeroy and Wiebe 1988), and bacteria utilising DOC may account for up to 50% of
oceanic production (Azam, Fenchel et al. 1983; Cole, Findlay et al. 1988). Mann and Wetzel (1995) found that the microbial loop was operating in a riverine wetland system, particularly in the surface waters. Enzyme studies in Australian floodplain wetlands (Boon 1989; Boon 1990; Boon and Sorrell 1991; Scholz and Boon 1993a; Scholz and Boon 1993b) support this finding. Bacterial production is known to be grazed by zooplankton in marine and freshwater systems (Calbet and Landry 1999; Sanders, Porter et al. 1989; Sherr and Sherr 2002; Sherr and Sherr 1991; Thorp and Delong 2002), and this links the microbial loop into the microbial food web and classical aquatic food chains (Lenz 1992).

Substances leached from submerged plant litter and soils are retained in the aquatic environment in the form of DOC in the water column and pore-water of the sediments. This DOC is comprised largely of humic and fulvic substances. The bio-available fraction, mainly low-molecular weight compounds, is rapidly metabolised by bacteria (Baldwin 1999; Findlay and Sinsabaugh 1999; Fischer, Mille-Lindblom et al. 2006; O’Connell 1998). Burns and Ryder (2001b) showed that microbial enzyme activity in river and wetland sediments increased sharply in response to pulses of DOC from flood waters. Where bacteria are most active, high molecular weight refractory DOC tends to accumulate, while the activity of fungi leads to the accumulation of DOC of intermediate molecular weight (Fischer, Mille-Lindblom et al. 2006). These refractory components of the DOC undergo photodegradation (Howitt, Baldwin et al. 2004; Moran and Zepp 1997), a process that tends to produce more biologically available carbon compounds. This may stimulate further microbial activity (Howitt 2003), but photodegradation is limited to the photic zone (Moran and Zepp 1997).

DOC is comprised largely (75%) of humic acids, with the remainder being composed of carbohydrates, fulvic acids, low molecular weight organic acids and amino acids (Hood, Williams et al. 2005; Thurman 1985) and is the dominant form of organic matter in the water column. When the water column is well oxygenated, both bacteria and fungi are capable of exploiting DOC, with phenyl oxidase producing fungi being particularly important in the degradation and transformation of aquatic humic substances (Claus and Filip 1998). However, fungal biomass in the water column is very low (Libkind, Brizzio et al. 2003; Wurzbacher, Bärlocher et al. 2010), and comprised mostly of yeasts and parasitic forms (Lefevre, Bardot et al. 2007). Thus, on the balance of available
information, bacteria are more important in utilising DOC in the water column of wetlands than fungi.

2.5.4 **Transformations of inorganic carbon**

Dissolved inorganic carbon is usually defined as the total concentration of carbon dioxide, carbonate and bicarbonate. In aquatic environments, transformations between these inorganic carbon species are highly dependent upon pH, and the chemical processes involved have been outlined in several texts (e.g. Manahan 1994). Carbon dioxide, bicarbonate and carbonate may also be reduced by methanogenic bacteria under anoxic conditions.

2.5.5 **Mineralisation of plant detritus**

In streams, particulate organic carbon (POC) is consumed by animals such as invertebrate shredders after first being colonised by micro-organisms including fungi and bacteria. According to the river continuum concept (Vannote, Minshall *et al.* 1980) detritus is processed sequentially by a series of invertebrate groups that utilise it in different ways. This system relies on the sequential processing of organic matter as it moves downstream with current. When not connected to the main channel, wetlands are not influenced by this unidirectional flow of carbon, so POC is consumed and decomposed *in situ*. Where wetlands are oligotrophic and aerobic, invertebrates may consume POC. However many wetlands suffer from anoxia, eutrophication and chemical extremes such as high salinity and low pH. In these cases, it is likely to be benthic micro-organisms that will utilise POC.

The oxic zone in wetlands usually consists of the water column and the top layer of the sediments (a few millimetres) (Alverez, Rico *et al.* 2001; Boon 2006; Buesing and Gessner 2006; Mitsch and Gosselink 1993a). The sediment associated with living plant roots will also be oxygenated. Aerobic micro-organisms require oxygen to act as an electron receptor in the oxidation of organic compounds, and as a reactant in the enzymatic breakdown of organic polymers (Brune, Frenzel *et al.* 2000; Reddy and DeLaune 2008). Also, when oxygen takes part in a redox reaction, a relatively high positive reduction potential is achieved.

For a single organism, aerobic decomposition of organic substances yields more energy than anaerobic decomposition because the organic substrates can be completely oxidised
to carbon dioxide rather than an intermediate compound. During the aerobic decomposition, complex carbohydrate polymers are cleaved by the extracellular enzymes of micro-organisms into monomers such as glucose. These are then transported into the organism’s cell where they undergo glycolysis and oxidative phosphorylation. This process yield 38 ATP molecules per glucose molecule (Lehninger, Nelson et al. 1993) and micro-organisms are able to capture approximately 30-50% of this energy. By way of comparison, the anaerobic fermentation of glucose to alcohol and carbon dioxide by yeasts yields 4 ATP molecules. The various end products of anaerobic carbohydrate metabolism may be degraded further by a series of anaerobic organisms using a range of metabolic pathways, ultimately yielding carbon dioxide and/or methane.

Fungi have been characterised as the most important group of micro-organisms responsible for the aerobic decomposition of plant material in agricultural soils, forest soils, freshwater sediments and peat lands (Collins, Sinsabaugh et al. 2008; Cornut, Elger et al. 2010; Freeman, Ostle et al. 2004; Gutknecht, Goodman et al. 2006; Wainwright 1992).

2.5.6 Mineralisation of sediment organic matter under oxic conditions

In wetlands, anoxic and oxic conditions can occur in extremely close proximity. Reduced oxygen diffusion into soils due to flooding, and high biological and chemical oxygen demands result in the development of 2 distinct soil/sediment layers; the oxidised surface layer where aerobic metabolic processes are possible and the reduced lower layer where respiration is anaerobic (Reddy and DeLaune 2008). Anaerobic respiration is carried out by sediment bacteria, but it is possible that fungi in the sediments, such as yeasts and chytrids, may generate energy by fermentation under these conditions (Wurzbacher, Bärlocher et al. 2010). The water column may also have an anoxic zone, particularly if temperatures, salinity or biological oxygen demands are high.

In hydrologically dynamic wetlands, such as floodplain wetlands that flood and dry, the vertical limit with an oxic zone above and an anoxic zone below varies in both space and time (Boon 2006), moving up and down with water levels. Plant roots and animal burrows can also create pathways through which oxygen can diffuse. Thus a mosaic of oxic/anoxic zones can develop in the sediments, and also in the water column, and may vary daily (Boon 2006).
POC on the sediment surface tends to be colonised by anaerobic decomposer organisms, which are predominantly bacteria. This is, in part, due to the high oxygen demand of wetland sediments (McLatchy and Reddy 1998).

In ephemeral systems, drawdown may lead to desiccation of sediment organic matter. As well as influencing microbial activity, desiccation can promote oxidative chemical reactions (Baldwin and Mitchell 2000). In the presence of molecular oxygen, carbon substrates are completely degraded to carbon dioxide via the aerobic respiration of bacteria, fungi, protozoans and wetland fauna. As oxygen is a very strong oxidant, almost all organic compounds represent possible substrates or food sources.

In the wetland environment oxygen is consumed in preference to other electron acceptors because aerobic metabolism releases more energy. Even organisms capable of utilising substrates via other metabolic pathways will use aerobic pathways if possible. Facultative anaerobes, capable of aerobic respiration and fermentation, are able to use the organic substrate itself as an electron acceptor in the absence of oxygen. However, not only is less energy obtained by fermentation, but it results in the production of a by-product that can not be further metabolised.

Twice the amount of carbon dioxide is generated by decomposer organisms under oxic conditions as under permanently anoxic conditions (Godshalk and Wetzel 1978a; Reddy and Patrick 1975). This gas accounts for most of the organic material decomposed under oxic conditions, with methane emissions being minor. As wetlands are inundated, there is a flush of inorganic carbon from the sediments, mostly in the form of carbon dioxide. With ongoing inundation, progressively more methane is evolved (Boon, Mitchell et al. 1997). This may be due to the decreasing availability of oxygen in the waterlogged sediments and water column due to biological and chemical processes, and the relatively poor solubility of oxygen in water, especially at elevated temperatures. Shifts from oxic to anoxic conditions in floodplain wetlands can occur rapidly (Boon and Mitchell 1995).

2.5.7 Mineralisation of sediment organic matter under anoxic conditions

Oxygen depletion is common in wetland sediments (Mitsch and Gosselink 1993a) and inside wood (Cooke and Whipps 1993). Where oxygen consumption exceeds oxygen
diffusion in the sediments, they will become anoxic. This change results in a shift in microbial metabolism from aerobic to anaerobic pathways. Under anoxic conditions, bacteria are considered to be responsible for the bulk of biologically mediated mineralisation processes, and fungi and protozoa are of minor importance.

While fungi are generally regarded as strictly aerobic heterotrophs, a number of studies have shown that they are able to survive, grow and participate in fermentation reaction under anoxic conditions. Fungi have been isolated from micro-aerobic conditions in litter (Reith, Drake et al. 2002), inside wood (Erickson, Blanchette et al. 1990b), and from blackened leaves in aquatic environments (Field and Webster 1983; Pattee and Chergui 1995).

Many fungal species are facultative anaerobes (Gibb and Walsh 1980). For example, the facultative anaerobic fungus *Aqualinderella fermentans* has been isolated from stagnant ponds and marshes that have low oxygen concentrations (Emerson and Natvig 1981). The alternating oxic/anoxic conditions common in wetlands may enable facultative anaerobes to store nutrients required for anaerobic metabolism during their aerobic growth phase (Cooke and Whipps 1993). Field and Webster (1983) demonstrated that fungi are able to survive under anoxic conditions in wetlands, but they are unable to grow.

Apart from lower energy yields, anoxic conditions cause oxidative enzymes to become inactive. This limits the range of substrates available for metabolism. Lignin, in particular, is mainly degraded via oxidative pathways. However, long term incubation experiments have revealed that lignin may be decomposed very slowly by anaerobic bacteria (Benner, Maccubbin et al. 1984). Moreover, the most reactive and bioavailable carbon is used in oxic zones (Hulthe, Hulth et al. 1998). The more refractory carbon that enters the anoxic zone must therefore be used at a slower rate (O'Connell, Baldwin et al. 2000).

Fermentation is carried out by either obligate or facultative anaerobes. Fermentation is the major anaerobic pathway whereby high molecular weight compounds are broken down into smaller compounds that can be utilised by other organisms (Mitsch and Gosselink 1993a; Reith, Drake et al. 2002; Van Laere 1994). Fermentative organisms
can utilise substrates such as organic acids, sugars, alcohols and amino acids. End products can include carbon dioxide, hydrogen, acetate, formate, butyrate, lactate, succinate, volatile fatty acids, and alcohols. These products may be further oxidised, but the process of oxidation will depend on the availability of electron receptors, the type of organic substrates available, and the microbes competing for the substrates. Further oxidation is carried out by denitrifiers, iron, sulfate and manganese reducers, and methanogens (Reddy and DeLaune 2008). In general, the process that yields the most energy from the oxidation of the organic substrate will dominate.

2.5.8 **Gaseous carbon emissions**

Wetlands may be a carbon source or a carbon sink (Mitsch and Gosselink 1993a). So the proportion of carbon flowing through the various decomposition pathways is important in determining the wetland’s net contribution to greenhouse gas emissions. As wetlands are generally open systems, the rates of organic matter import, export and storage are also significant.

Occasionally in some wetlands, a balance between methanogenesis and methanotrophy results in negligible methane emissions (Boon, Mitchell et al. 1997). Boon (2000) has discussed the importance of methanogenesis, methanotrophy and methane emission in wetland carbon cycles. He reported that methane emissions from a range of different floodplain wetlands in Australia ranged from the limits of detection to 8mmol.m$^{-2}$.hr$^{-1}$. Emissions from permanent natural wetlands showed high variability, with winter maxima below the limit of detection and summer maxima reaching 3 mmol.m$^{-2}$.hr$^{-1}$ (Boon 2000; Sorrell and Boon 1992a). Methane emissions were also high in areas supporting emergent macrophytes, and daytime emissions were double nightly rates. Emissions from ephemeral wetlands fell within the same range of values as permanent wetlands, but were dependent on the timing and duration of flooding (Boon 2000). Occasionally in some wetlands, a balance between methanogenesis and methanotrophy resulted in negligible methane emissions (Boon, Mitchell et al. 1997). Boon (2000) has estimated that the bulk of carbon emissions from wetland sediments are the result of methanogenesis, with aerobic decay having a secondary role in gas emission.

In summer months, the water column in many shallow floodplain wetlands becomes stratified with respect to oxygen concentration (Ford, Boon et al. 2002). As a result,
surface waters are oxygenated but contain virtually no methane during the day, while de-
oxygenated water overlying the anoxic sediments is high in methane. This situation
places severe restriction on the oxidation of methane by methanotrophic bacteria (Ford,
Boon et al. 2002). However, nightly overturn of the water column mixes the stratified
layers and promotes methanotrophy in the early morning. This suggests that stratification
is an important factor contributing to elevated methane emissions where shallow
wetlands hold water over the summer period.

2.6 Plant detritus as a carbon resource
The rate at which plant detritus is decomposed, and the micro-organisms that mediate
decomposition, are strongly influenced by both the chemical composition of the substrate
and the distribution of the substrate through space and time. Simple and easily
metabolised plant carbohydrates such as sugars typically occur at low concentrations in
the environment because they are rapidly turned over by common decomposer micro-
organisms (Suberkropp and Klug 1976; Tranvik, van Hees et al. 2004). More complex
compounds, such as the structural carbohydrates, build up in the environment as a result
of extensive biogeochemical transformations (Suberkropp and Klug 1976). They resist
rapid microbial decomposition and are not generally known or described as discrete
molecules (Tranvik, van Hees et al. 2004).

2.6.1 Plant structural carbohydrates
Structural carbohydrates of plant origin include cellulose, hemicellulose and lignin.
These are complex polymers composed of repeating sub-units of sugars in cellulose and
hemicellulose, and phenyls in lignins. In order to be utilised metabolically, these
polymers must be broken down enzymatically.

2.6.1.1 Cellulose
Cellulose is a linear polymer composed of around 14 000 repeating β-1,4-glucose
residues, with cellobiose (a disaccharide) being the basic repeating unit. It is the most
abundant organic resource on Earth, with approximately 4 x 10^9 tons being formed
annually (Aro, Pakula et al. 2005; Melillo, McGuire et al. 1993). Cellulose comprises 30-
50% of plant cell walls (Esau 1977) and 40-50% of the dry weight of temperate woods
(Erickson, Blanchette et al. 1990c; Shearer 1992). In wetlands, 10-20% of leaf litter and
40-50% of mature wood is composed of cellulose (Boulton and Boon 1991).
As a component of plant cell walls, cellulose is found in association with pectin and hemicellulose, and it is additionally cross-linked to lignin in structural tissues (Evert and Esau 2006). The proportion of cellulose in leaf material remains fairly constant throughout decomposition, paralleling mass loss (Suberkropp and Klug 1976). This may be due to its gradual exposure to microbial activity as lignin is slowly removed. It may be that cellulose and hemicellulose are metabolised preferentially, and that lignin decomposition is a secondary metabolic process triggered by nutrient limitation employed primarily as a means of further exposing cellulose (Cooke and Whipps 1993; Cullen and Kersten 1992; Suberkropp, Godshalk et al. 1976; Van Laere 1994).

2.6.1.2 Hemicellulose

Hemicellulose is the second most abundant polysaccharide in nature and generally takes the form of an heterogeneous polymer composed of beta-linked sugar units. These sugars include β-1,4-D-xylans, β-1,4-D-mannans, and β-1,4- and β-1,3-galactans. The xylans are most common in cereals and hard woods and have a backbone composed of β-1,4-linked D-xylose. (Galacto)glucomannans are found in soft woods and hard woods and have a backbone of β-1,4-linked D-mannose. Arabinan and galactan are less abundant, and consist of α-1,5-linked L-arabinose and β-1,3-linked D-galactose units respectively (Aro, Pakula et al. 2005).

The percentage of hemicellulose in leaf material has been found to remain fairly constant over the period of decomposition, paralleling mass loss (Suberkropp, Godshalk et al. 1976). It is degraded at a rate equal to, or faster than, cellulose decomposition in most wetlands, and is degraded five times faster than lignin (Suberkropp, Godshalk et al. 1976). The rate of hemicellulose decomposition is lowered by anoxic conditions (Ford 1993).

2.6.1.3 Lignin

Lignins are complex aromatic polymers formed from phenyl propanoid subunits, such as coniferyl, synapyl and ρ-coumaryl alcohols, joined by carbon-carbon and ether linkages. In cell walls, they are also extensively cross-linked (Aro, Pakula et al. 2005). Strong and random bonding between phenyl-propane subunits, and the diversity of chemical structures found in lignins from different plants, make this polymer resistant to microbial degradation (Dix and Webster 1995).
Lignins comprise 20-30% of woody tissue and wetland macrophytes (Ford 1993; Kirk and Farrell 1987; Shearer 1992), and 10% of leaf litter mass (Suberkropp, Godshalk et al. 1976). They form a physical barrier that protects cellulose and hemicellulose from enzymatic attack (Aro, Pakula et al. 2005).

The lignin content of submerged wood is a strong influence on its decay rate (Melillo, Naiman et al. 1984). In leaf material, lignin content has been found to increase over the period of aquatic decomposition. Moreover, the absolute magnitude of the lignin fraction in submerged leaves also increases, remains stable for a time and then subsequently decreases (Suberkropp, Godshalk et al. 1976). Suberkropp et al. (1976) suggested that this increase in total lignin content was a result of the formation of complexes composed of lignin and nitrogen compound.

2.7 Fungal Ecology

It is well established that fungi are important in the decomposition of dead plant material under terrestrial (Swift, Heal et al. 1979) and aquatic (Gessner, Gulis et al. 2007) conditions. In many forest ecosystems, fungi dominate the microbial biomass in both the litter and soil layers (Cromack and Caldwell 1992; Eicker 1973; Kjoller and Struwe 1992; Wainwright 1992). In these systems they are fundamental to the transformation and translocation of complex detrital molecules (Cromack and Caldwell 1992).

As multicellular decomposer organisms, fungi have several metabolic advantages over bacteria. They are capable of indeterminate growth and can spread throughout large areas; they are able to translocate nutrients and metabolites through their mycelia; and fungi are able to tolerate stressful environmental conditions (Jennings and Lysek 1996). Regardless of these advantages, the decomposer community is usually comprised of fungi, bacteria, protozoa and invertebrates (Schulze and Walker 1997). It is the interaction of these organisms that determines the rate and mechanisms of plant detritus break down and decomposition (Hieber and Gessner 2002; Mille-Lindblom, Fischer et al. 2006a).

Fungi are able to colonise a wide range of substrates rich in organic compounds. These include living plants and animals, dead plants and animals, soil organic matter and dissolved organic matter. There is also evidence that fungi are capable of fixing carbon
dioxide from the atmosphere (Parkinson, Wainwright et al. 1989). Fungi have been found to degrade plant detritus in aquatic environments including salt marshes (Buchan, Newell et al. 2003; Weinstein, Kreeger et al. 2002), freshwater marshes (Gessner and Van Ryckegem 2003; Kuehn and Suberkropp 1998b), peat bogs (Thormann, Currah et al. 2003; Trinder, Johnson et al. 2008) and streams (Cornut, Elger et al. 2010; Gulis, Kuehn et al. 2009; Hieber and Gessner 2002; Pascoal, Cássio et al. 2005). The aquatic hyphomycetes in particular, are of high importance in the breakdown of leaf litter in streams (Bärlocher 1992; Bärlocher, Canhoto et al. 1995; Bärlocher and Graça 2002; Graça, Pozo et al. 2002; Sridhar, Duarte et al. 2009; Suberkropp 2001). The importance of fungi in floodplain wetlands has not yet been established.

There is a limited amount of research into the ecology of fungi in Australian freshwater wetlands (Scholz and Boon 1993b) and rivers (Suter 2009; Swart 1986; Thomas 1992; Thomas, Chilvers et al. 1989; Thomas, Chilvers et al. 1992). However, over the last decade, advances have been made in techniques for detecting and characterising fungal populations (e.g. Andjic, Hardy et al. 2007; Borneman and Hartin 2000; Boulton and Boon 1991; Collado, Platas et al. 2007; Fackler, Schwanninger et al. 2007; Glen, Tommerup et al. 2001; Guglielmo, Bergemann et al. 2007; Kumar and Shukla 2005; Lawley, Ripley et al. 2004; Martin and Rygiewicz 2005; Ritz 2007; Rodriguez, Cullen et al. 2004; Singh, Nazaries et al. 2006). These new techniques now provide the means to further investigate the contribution of the fungi to floodplain carbon cycling.

2.7.1 **Fungi as decomposers of plant material**

Saprotrophic fungi (saprobes) are fungi that obtain their nutrition from dead organic matter. The organic matter is known as their “substrate”, and serves as both habitat and food source. Fungi invade the plant tissue and obtain nutrition by secreting extracellular enzymes. These enzymes break down plant polysaccharides into monosaccharides that can be readily absorbed across the fungal cell wall. Not all plant cell components can be broken down and absorbed by all fungal species, although it is a competitive advantage to be able to use a range of carbon sources (Wainwright 1992). For this reason most species of fungi elaborate a range of different extracellular enzymes, but the suite of enzymes produced will differ between species. The enzymes that a species produces will dictate which substrates it can successfully colonise.
2.7.2  **Aquatic fungi**

The fungi known to inhabit stream, lakes and wetlands include representatives of the Eumycota (true fungi), Stramenopiles (fungus-like protists) and Labyrinthulomycetes (slime moulds). Within the Eumycota, all major phyla are represented (Ascomycota, Chytridiomycota, Zygomycota and Basidiomycota) (Nikolcheva and Bärlocher 2004; Shearer, Descals et al. 2007). Saprotrophic fungi found colonising submerged wood and leaves include the aquatic hyphomycetes and aero-aquatic hyphomycetes. These are functionally defined groups. The aquatic hyphomycetes reproduce via sigmoid or tetra-radiate conidia (Ingold 1976), while aero-aquatic hyphomycetes reproduce via buoyant conidia (Premdas and Kendrick 1991). Stramenopiles are represented by the Oomycetes, which reproduce asexually via flagellated zoospores (Dick 1969). Aquatic orders include the Saprolegniales, Leptomitales, Lagenidales and Peronosporales (Dick 1976b). Labyrinthulomycetes are not generally considered as saprotrophic fungi, and they are not addressed further in this thesis.

Gulis et al. (2009) recently discussed the role of fungi in fresh water environments, while Jobard et al. (2010) reviewed the importance of fungal organisms in pelagic food webs and Wurzbacher et al. (2010) reviewed the role of fungi in lake ecosystems.

2.7.2.1  **Aquatic hyphomycetes**

The aquatic hyphomycetes are a sub-group within the Deuteromycotina, a group defined by the absence of a sexual life stage (Hawksworth and Rossman 1997). The name “hyphomycetes” is derived from the fact that they produce conidia directly from the mycelium, not from a fruiting body. Genetically, the majority of aquatic hyphomycetes are classified with the Ascomycota, but some are members of the Basidiomycota. Aquatic hyphomycetes are the dominant fungal group on tree leaves in fast flowing streams (Bärlocher 1992; Ingold 1942) and their spores are adapted to rapidly disperse longitudinally in the stream (Iqbal and Webster 1973). These fungi are absent in silted or muddy areas where leaves turn black due to lack of oxygen. However, they may be found in terrestrial habitats (Webster 1977), or colonising living plants in streams (Sati and Belwal 2005; Sridhar and Bärlocher 1992).

2.7.2.2  **Aero-aquatic hyphomycetes**
Aer-aquatic fungi are a functionally defined group that are most abundant in shallow, stagnant waters (Field and Webster 1983; Fisher and Webster 1979) where they produce vegetative growth on submerged plant litter. They are also known to occur in streams (Bärlocher 1992; Goos 1987; Willoughby and Archer 1973). Sporulation only occurs when the substrate becomes exposed to air (Pattee and Chergui 1995; Shearer, Descals et al. 2007) and the resultant propagules are buoyant and float on the water’s surface. Taxonomically, most aer-aquatic fungi are classified as mitosporic Ascomycetes (Ascomycetes lacking a sexual phase in development). Four aer-aquatic species have been classified as Basidiomycetes, and one as Oomycete (Shearer, Descals et al. 2007).

2.7.2.3 Oomycetes

The Oomycetes (Peronosporomycetes), also known as water moulds, are aquatic organisms common in waterlogged soils (Dick 1962; Dick 1963) and stagnant waters such as ditches, ponds (Park, Sorenson et al. 1978), the littoral zones of lakes (Dick 1976b; Hallett and Dick 1981; Nechwatal, Wielgoss et al. 2008), wetlands (Czeczuga, Muszyńska et al. 2007), and streams (Shearer and von Bodman 1983). Common genera include Achlya, Saprolegnia, Pythium, Phytophthora, Aphanomyces, Dictyuchus and Leptolegnia (Hallett and Dick 1981; Nechwatal, Wielgoss et al. 2008; Park, Sorenson et al. 1978). The Achlya and Saprolegnia are abundant in near-shore areas, while Pythium and Aphanomyces are found in oxic pond sediments (Park, Sorenson et al. 1978). However, the abundance of Oomycetes and their propagules is usually much lower in sediments than in surface waters (Hallett and Dick 1981).

The Oomycetes are taxonomically distinct from the Eumycota (true fungi), being more closely related to brown algae and diatoms and possibly evolved from a photosynthetic ancestor (Lamour, Win et al. 2007). Morphologically, however, they are similar in form and habitat to the Chytridiomycetes (see below). When colonising submerged plant litter, Oomycetes are observed early, then rapidly decline (Bärlocher 1990; Bärlocher and Kendrick 1974). Peaks in the abundance of Oomycete propagules have been observed to coincide with the seasonal influx of fresh substrate in spring, summer and autumn (Hallett and Dick 1981), further suggesting a preference for newly submerged litter. These organisms prefer neutral to acid pH (Dick 1963), and may be limited by calcium concentration (Czeczuga, Muszyńska et al. 2007), which is a cofactor for the enzyme pectin lyase (Suberkropp 2001).
Many species of Oomycetes are pathogenic to plants and animals. This includes *Pythium phragmitis*, which is known to infect the common wetland reed, *Phragmites australis* (Nechwatal, Wielgoss *et al.* 2008). Most of the species belonging to *Achlya*, *Dictyuchus* and *Saprolegnia* are opportunistic pathogens to fish (Leclerc, Guillot *et al.* 2000), taking advantage of minor injuries to infect surface tissues (Singhal, Jeet *et al.* 1987). They may also infect crustaceans, mosquito larvae and the eggs of fish and invertebrates (Hulvey, Padgett *et al.* 2007).

### 2.7.2.4 Chytrids

Chytrids (Chytridiomycota) are an important component of the fungal decomposer community in fresh water environments. They are also parasites of both plants and animals, able to digest proteins, chitin, and cellulose with high efficiency. Chytrids reproduce asexually via the production of zoospores. These zoospores may be an important food source for zooplankton. The ecology of Chytrids in aquatic ecosystems is reviewed by Gleason *et al.* (2008).

### 2.7.3 Fungal communities

While more than 600 species of fungi are known to colonise the leaves of *Phragmites australis* (Gessner and Van Ryckegem 2003), less than twenty species generally coexist on the substrate at any one time. Three processes interact to determine which species will be successful. Firstly, their ability to use the available resources (i.e. the enzymes they can secrete) (Chamier and Dixon 1982b). Secondly, environmental factors such as temperature, water flow and oxygen availability (Findlay and Arsuffi 1989; Pattee and Chergui 1995) select for a pool of species that are tolerant of local conditions and can potentially colonise a new substrate. Thirdly, competition for resources on a discrete substrate such as a submerged leaf initiates a succession of species over time (Fryar, Booth *et al.* 2005; Mille-Lindblom, Fischer *et al.* 2006a).

The fungal communities on submerged litter substrates in streams are usually composed of four to ten abundant or common species of aquatic hyphomycetes (Chamier, Dixon *et al.* 1984; Thomas, Chilvers *et al.* 1992). These communities vary seasonally (Nikolcheva and Bärlocher 2005; Thomas, Chilvers *et al.* 1989). In Australia, distinct communities colonise the submerged leaves of *Eucalyptus viminalis* in summer/autumn and spring/summer, although some species show no seasonal variability (Thomas, Chilvers *et al.* 2007).
al. 1992). While seasonal changes in fungal community composition may be correlated with seasonal litter inputs (Thomas, Chilvers et al. 1989), Suberkropp (1984) found that many species were found predominantly in summer or winter regardless of the time litter was introduced to the stream.

The communities of aquatic fungi found in flowing and standing water habitats are distinctive (Wong, Goh et al. 1998). Broadly speaking, flowing waters are dominated by aquatic hyphomycetes, while standing waters are dominated by aero-aquatic hyphomycetes (Pattee and Chergui 1995). Many species of aero-aquatic fungi are also able to withstand prolonged periods of drying (Field and Webster 1983), which is advantageous in ephemeral habitats such as floodplain wetlands. Standing water has lower oxygen concentration, higher temperatures, and higher concentrations of nutrients and DOC relative to flowing water and wetland fungi tend to be adapted to these conditions.

Reduced oxygen availability will select species able to metabolise carbon anaerobically (fermentation), disadvantaging species that oxidise lignin. Fungi are able to ferment glucose (Cochrane 1956), starch (Oda, Saito et al. 2002) and gelatine (Roberts 1899), and grow anaerobically (Davies, Theodorou et al. 1993; Theodorou, Lowe et al. 1992; Trinci, Davies et al. 1994), but anaerobic fungi comprise a small proportion of anaerobic saprobes in terrestrial litter (Reith, Drake et al. 2002). The metabolic pathways used by fungi under aquatic anoxic conditions require investigation. However, many aero-aquatic hyphomycetes are able to survive anoxic conditions for up to 12 months as vegetative hyphae (Field and Webster 1983), although they may not grow under these conditions (Fisher and Webster 1979). Aquatic hyphomycetes can survive for limited periods under low oxygen conditions (Field and Webster 1983) but they experience reduced levels of function in terms of leaf decomposition, diversity, biomass and reproduction (Medeiros, Pascoal et al. 2009b). Pattee and Chergui (1995) found a gradient from the well-oxygenated waters of the Rhone River where aquatic hyphomycetes were dominant, to the anoxic conditions of an ox-bow lake on the Rhone floodplain where aero-aquatic hyphomycetes dominated.

Leaf species is often the most important controlling factor in the composition of fungal decomposer communities (Thomas 1992; Thomas, Chilvers et al. 1992). Gulis (2001)
found that at least some species show preference for specific substrates, with communities on wood and grass being distinct from those found on tree leaves. The chemical composition of the substrate differs between species, and will also change as decay progresses. The proportion of cellulose, hemicellulose and plant defensive compounds decreases with decay, while the proportion of lignin increases (Golladay and Sinsabaugh 1991; Rosset, Bärlocher et al. 1982; Suberkropp, Godshalk et al. 1976). This confers an advantage on species able to degrade lignin, and encourages a change in community composition. As the substrate becomes more fragmented the decomposer community becomes increasingly dominated by bacteria (Baldy, Gessner et al. 1995; Golladay and Sinsabaugh 1991; Suberkropp and Klug 1976).

Mille-Lindblom et al. (2006b) found that substrate quality was more important than water chemistry in regulating the composition of the microbial community on submerged litter in lakes, while Fischer et al. (2009) found water chemistry had the stronger influence on stream communities. Schoenlein-Crusius et al. (1999) found that the occurrence of aquatic hyphomycetes, and to a lesser extent zoosporic fungi, was correlated with high concentrations of calcium in the substrate.

Competition is an important structuring agent for all ecological communities, including decomposer communities (Fryar, Booth et al. 2005; Mille-Lindblom, Fischer et al. 2006a; Mille-Lindblom and Tranvik 2003; Wicklow 1981). For example, under aquatic conditions, terrestrial fungi do not generally compete successfully with aquatic hyphomycetes (Bärlocher and Kendrick 1974; Suberkropp and Klug 1976). This is likely to be because they are less efficient at acquiring and metabolising resources under these conditions. However, some species of aquatic hyphomycetes have been shown to produce antibiotic substances that are able to inhibit the growth of bacteria and other fungi (Gulis and Stephanovich 1999). Conversely, bacteria (Fischer, Mille-Lindblom et al. 2006; Moller, Miller et al. 1999; Romani, Fischer et al. 2006), invertebrates (Bärlocher and Kendrick 1975) and viruses (Jackson and Jackson 2008) may inhibit fungal growth, and bacteria may benefit from the presence of fungi (Romani, Fischer et al. 2006).

Colonisation of aquatic detritus is random and patchy, and the composition of the fungal community on submerged litter may be difficult to predict (Fischer, Bergfur et al. 2009).
On substrata introduced into streams, species richness is initially low, rises to a peak (within weeks or days) then remains stable or declines (Nikolcheva, Cockshutt et al. 2003). Those fungal species that are first to colonise a substrate have a strong influence over the composition of the fungal community that subsequently develops (Bärlocher 1992; Sanders and Anderson 1979). This effect remains in place even if environmental conditions change markedly (Sridhar, Duarte et al. 2009). However a change from aerial to aquatic conditions leads to a gradual change in community composition (Van Ryckegem, Gessner et al. 2007).

2.7.4 Biomass

The abundance of fungal organisms on submerged plant substrates is generally measured in terms of biomass. This is commonly estimated by determining the content of the fungal biomarker ergosterol in the leaf matrix. Biomass is influenced more strongly by substrate type than is community structure (Nikolcheva and Bärlocher 2005), but it is also influenced by water quality parameters (Sridhar and Bärlocher 2000; Suberkropp and Chauvet 1995).

When new substrates enter the aquatic environment, fungal biomass tends to steadily increase to a maximum and then decline (Das, Royer et al. 2007). The time taken to reach maximum biomass may vary with leaf species and environmental conditions such as nitrate concentrations (Das, Royer et al. 2007). During the period leading up to “full conditioning”, fungal biomass generally exceeds bacterial biomass (Findlay and Arsuffi 1989; Kaushik and Hynes 1971) and sporulation and mycelial production continue even as fungal biomass declines (Baldy, Gessner et al. 1995).

Where a leaf substrate has been colonised by fungi in a terrestrial or aerial environment, fungal biomass may decrease markedly when the substrate is submerged. Fungal biomass on decaying standing leaf blades of Phragmites australis reached 548 μg ergosterol /g AFDM, but decreased to about one third of that value (191 μg ergosterol/g AFDM) when the leaf blade collapsed into brackish marsh water (Van Ryckegem, Gessner et al. 2007).

2.7.5 Enzyme activity

The synergistic activity of microbial extracellular enzymes is the main driver of plant litter decomposition. While no single enzyme can be used to predict litter mass loss, multiple linear regression of enzyme activity shows that mass loss is strongly related to
the combined activity of a range of ligno-cellulose degrading enzymes (Jackson, Foreman et al. 1995; Sinsabaugh, Moorhead et al. 1994). This implies that the rate of litter decomposition is controlled by the same factors that influence enzyme activity. These factors include the chemical composition of the substrate, and environmental conditions such as leaching, temperature and pH (Chróst 1990).

Soluble organic matter is leached from plant litter within days of immersion (See 2.5.3). Thus, only the more refractory components of the middle lamella and primary and secondary cell walls (Chamier 1985) are available to fungi after leaching. The middle lamella is rich in pectic polysaccharides, while the primary cell wall is composed of cellulose, hemicellulose, pectic polysaccharides and glycoprotein. The secondary cell wall is mainly composed of cellulose but does not contain pectic compounds. If lignification occurs, it begins in the middle lamella and proceeds through the primary and secondary cell walls (Evert and Esau 2006). Thus, enzyme activity on submerged litter is primarily towards cellulose and hemicellulose, as these compounds are most abundant. Activity towards lignin, pectin and minor plant components will also be present. While the rate of fungal mineralisation of organic substrates is governed by the production of enzymes, enzyme production is controlled by the availability of nitrogen and phosphorus. Where nutrients become limiting, fungi are required to expend energy to use enzymes to sequester more nutrients from their substrate. Thus, enzyme expression is largely controlled by the substrate quality (Sinsabaugh, Gallo et al. 2005).

Enzyme activity may be influenced by temperature, pH and salinity (Chróst 1990). It is well established that increases in temperature stimulate enzyme activity (Sinsabaugh, Moorhead et al. 1994). Thus, optimum temperatures for enzyme activity are usually well above those occurring in natural aquatic environments, so many microbes show no adaptation to temperature (Chróst 1990). However, pH varies widely between aquatic ecosystems, and microbes show preference for acid or alkaline pH ranges similar to those occurring in natural systems, 4.0-5.5 and 7.5-8.5 respectively (Chróst 1990). Enzymatic litter decomposition is also known to be inhibited by salinity. In marine environments, both low and high salinities are inhibitory (King 1986).

Aquatic hyphomycetes are able to secrete enzymes that are active towards all the major leaf structural polysaccharides (Abdullah and Taj-Aldeen 1989; Suberkropp, Arsuffi et

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al. 1983), although the level of activity varies with species (Chamier 1985). Two-thirds of aquatic hyphomycetes and aero-aquatic hyphomycetes examined by Abdullah and Taj-Aldeen (1989) were able to hydrolyse cellulose. Aquatic hyphomycetes are able to hydrolyse hemicellulose (Chamier 1985; Thornton 1963) and oxidise lignin (Abdel-Raheem and Shearer 2002; Abdel-Raheem and Ali 2004). The Basidiomycetes are well recognised for their importance in lignin degradation in terrestrial plant litter (Cromack and Caldwell 1992; Mackensen, Bauhus et al. 2003) but they have not been proven to actively decompose litter under aquatic conditions.

### 2.7.6 Enzymatic breakdown of plant polymers by fungi

Virtually all fungal species are able to readily absorb naturally occurring low molecular weight carbohydrate monomers. Most fungi are able to enzymatically degrade organic polymers, such as protein, pectin, cellulose and starch, to monomers and thence to carbon dioxide and methane. Fewer species are also able to degrade lignin (Dix and Webster 1995). The following is a brief summary of the mechanisms involved in the enzymatic breakdown of the major plant cell wall polymers. The decomposition of plant litter in aquatic environments by fungi has been thoroughly reviewed by Gessner et al. (2007), and Gamauf et al. (2007) discuss the fungal degradation of plant cell wall polymers.

#### 2.7.6.1 Cellulose

A minimum of three enzymes are required to degrade cellulose completely. These enzymes act synergistically to cleave glycosidic bonds to produce smaller chains, cellobiose dimers and finally glucose (Ander 1994; Erickson, Blanchette et al. 1990a). Endoglucanases (endocellulases) are usually secreted first. These are enzymes that cleave internal β-1,4-linkages, to produce oligosaccharides. Cellobiohydrolases (exocellulases) simultaneously cleave terminal β-1,4-linkages from either the reducing or non-reducing end of the oligosaccharide, depending on the nature of the enzyme. This liberates cellobiose. Finally, β-glucosidases attack oligosaccharides and cellobiose to remove D-glucose (Aro, Pakula et al. 2005; Gamauf, Metz et al. 2007). Most saprotrophic fungi will produce a range of cellulose degrading enzymes. For example, the soft rot fungus *Trichoderma reesei* produces glycoside hydrolases belonging to seven enzyme families (Warren 1996).

Cellulose degrading enzymes may also be oxidative (Ander 1994; Ayers, Ayers et al. 1978). For example, cellobiose oxidase uses molecular oxygen to oxidise cellobiose and
oligosaccharides to give gluconic and cellubionic acids (Ljungdahl and Eriksson 1985). An alternative pathway for cellulose oxidation has been observed in *Sporotrichum pulverulentum*. Cellulose:quinone oxidoreductase (cellulose dehydrogenase) reduces quinones and phenoxy radicals (from lignin degradation) in the presence of cellulobiose (Ayers, Ayers et al. 1978). These systems are common in white rot fungi. Brown rot fungi may attack cellulose via a hydrogen peroxide:ferric iron system (Shimada, Akamtsu et al. 1997) that cleaves off reducing sugars in the presence of oxalic acid.

The synthesis of cellulases is generally repressed when glucose or other readily metabolised sugars are present. Lignin-related phenols may also repress cellulase production. In this case, phenol oxidases (ligninases) may polymerise these phenols to reduce the inhibitory effect (Ljungdahl and Eriksson 1985).

2.7.6.2 Hemicellulose

As hemicellulose is a heterogeneous polymer a range of enzymes is required to hydrolyse it completely. A suite of enzymes generally works in concert, as follows. Firstly, exoglycosidase removes side chains, and then endohemicellulase attacks the main glycan chain at unbranched points. This reaction produces oligosaccharides. Exoglycosidase and hemicellulase then cleave the oligosaccharides into monosaccharides, which are rapidly taken up through the fungal cell wall. For example, the degradation of xylan involves endo-1,4-β-D-xylanases and β-xylosidases, which attack the sugar chain forming the back bone of the polymer. Side chain cleaving enzymes may include α-glucuronidase and acetyl xylan esterase (Aro, Pakula et al. 2005; de Vries and Visser 2001). Alternative enzyme systems, which act via similar mechanisms, are required to degrade xyloglucan and galacto-glucomannan (Gamauf, Metz et al. 2007). The presence of short-chain oligo-polymers of xylose will induce the secretion of these enzymes (Cooke and Whipps 1993).

2.7.6.3 Pectin

Pectin is comprised of polymerised α-1,4-linked galactouropyranose, which may have methylated carboxyl groups. It may also contain α-1,2-linked rhamnose, α-1,3-linked arabinofuranose (araban), α-1,5-linked arabinofuranose and β-1,4-linked galactopyranose (galactan) (Cooke and Whipps 1993). Pectin degrading enzymes are hydrolytic and include polygalacturonases and pectin lyases. Pectate lyases, which act on non-esterified pectin, are characteristic of bacteria. Aquatic hyphomycetes exhibit polygalacturonase.
activity at pH values above 5 (Chamier and Dixon 1982a; Chamier and Dixon 1982b). The uptake of the released galacturonic acids stimulates fungi to release further enzymes including hemicellulases and cellulases (Cooke and Whipps 1993). Conversely, high concentrations of sugars can repress the production of these pectinases (Cooke and Whipps 1993). Pectin degrading enzymes are the first to be induced on a plant based substrate. Pectinase activity upon the middle lamella can separate cells and expose cell walls to further degradation.

2.7.6.4 Lignin

Lignin is highly resistant to hydrolytic degradation, so lignin degrading enzymes are oxidative, rather than hydrolytic. White rot Basidiomycetes are the only microbial group able to mineralise lignin to carbon dioxide and water. Brown rot fungi such as the xylariaceous Ascomycotina modify lignin by demethylation but cannot degrade it completely. Soft rot fungi attack lignin under water logged or dry conditions (Gamauf, Metz et al. 2007).

In lignified plant tissues, lignin must be degraded before energy can be obtained from polysaccharides. Lignin degradation is thought to be a secondary metabolic function for most fungal species, serving to expose cellulose (Cooke and Whipps 1993). Other research suggests that lignin degrading enzymes are induced by conditions of nitrogen starvation. Either way, lignin breakdown does not produce enough energy to be self-sustaining (Jeffries, Choi et al. 1981), so expenditure of cellular energy to degrade it is likely to be a means for obtaining additional energy or nutrients.

As lignin is a complex and heterogeneous aromatic polymer, numerous chemical reactions would be required to complete its breakdown. A range of breakdown products may also be degraded (Ljungdahl and Eriksson 1985). The main fungal enzymes involved in lignin degradation are laccases (phenol oxidases), manganese peroxidases and lignin peroxidases. Non-proteinaceous compounds, low molecular weight free radicals, are released into the substrate to initiate decay in many cases. Additional enzymes may be secreted to generate these molecules (Aro, Pakula et al. 2005).

Phenol oxidases oxidise benzenediols to semi-quinones with molecular oxygen. Laccases (benzenediol: oxygen oxidoreductase) are enzymes belonging to this group, that contain
copper at their catalytic centre and are active against phenols and similar molecules. They oxidise polyphenols, methoxy-substituted phenols and aromatic di-amines by reducing molecular oxygen to water (Gamauf, Metz et al. 2007). Laccase, in the presence of suitable substrates may also oxidatively cleave carbon-carbon linkages in lignin and oxidise veratryl alcohol to veratraldehyde (Bourbonnais and Paice 1990).

Laccases are commonly produced by Basidiomycetes, and may be produced by Ascomycetes. There are no reports indicating that Zygomyces or Chytridiomycetes produce laccases (Baldrian 2006). In water-saturated environments, laccase activity is driven by the availability of oxygen. For example, laccase activity is generally low in peatlands (Pind, Freeman et al. 1994; Williams, Shingara et al. 2000), but when oxygen becomes available as a result of drawdown, laccase activity increases sharply. Increased laccase activity can lead to the depletion of compounds that inhibit the activity of other oxidative and hydrolytic enzymes (Freeman, Ostle et al. 2004). Under water, laccase may be involved in the degradation of wood and humic substances (Claus and Filip 1998; Hendel and Marxsen 2000).

Peroxidases are a group of enzymes which catalyse the oxidation of substrate by a peroxide to give phenols. Lignin peroxidases resemble other peroxidases in that they contain a heme group and operate via a catalytic cycle. That is, the enzyme is oxidised by hydrogen peroxide and two electrons are removed. The enzyme returns to its resting state by oxidising donor substrate, removing one electron from each of two molecules. These substrates usually include phenol, anilines, aromatic ethers and non-phenolic lignin structures (Hammel 1997).

Manganese peroxidases are similar to other peroxidases, except that Mn(II) is the electron donor (Mn(III) is generated). The reaction requires the presence of organic acid chelators such as glycolate or oxalate, which stabilise Mn(III) and allow it to be released from the enzyme. These Mn(III) chelates oxidise the more reactive structures that make up about 10% of the lignin polymer. The activity of this enzyme on lignin may facilitate subsequent attack by lignin peroxidase (Hammel 1997).

Fungi may also secrete additional compounds such as hydrogen peroxide and veratraldehyde, which diffuse into the substrate away from the hyphae and penetrate into
the ligno-cellulose. These substances may initiate degradation. Brown rot fungi initiate 
lignin degradation by producing low molecular weight diffusible compounds that are 
able to act upon the wood at a distance from the hyphae. These compounds might include 
hydroxyl, peroxyl and hydro-peroxyl radicals, which are generated via the Fenton 
reaction (Gamauf, Metz et al. 2007). The presence of molecular oxygen and iron are 
required for this reaction to proceed. Many fungi also secrete oxalic acid, which chelates 
iron, but also reduces the pH, increasing enzyme activity (Gamauf, Metz et al. 2007).

Where peroxidases are active, a source of hydrogen peroxide is required to initially 
oxidise the enzyme. This is achieved by extracellular oxidases which reduce molecular 
oxygen to hydrogen peroxide while oxidising low molecular weight organic compounds. 
Similar compounds may act as intermediaries in substrate oxidation. For example, 
veratryl alcohol can be oxidised to a radical cation which is not rapidly degraded. It can 
thus act as a one-electron oxidant when it encounters a second oxidisable substrate, and 
in the process the veratryl alcohol molecule is regenerated (Harvey, Schoemaker et al. 
1986).

Given that lignin degradation is an oxidative process, lignin has previously been 
considered to be inert under anoxic conditions (Evans and Fuchs 1988; Kirk and Farrell 
1987). However, long term incubation of wood blocks in anaerobic mud revealed that 
they were slowly degraded by rod-shaped bacteria (Benner, Maccubbin et al. 1984; Holt 
and Jones 1983). It has also been believed that aquatic hyphomycetes were not able to 
degrad lignin (Boulton and Boon 1991). However, both aquatic and aero-aquatic 
hyphomycetes are capable of partial lignin break down (Fisher and Webster 1979). Thus 
aquatic lignin degradation by fungi is plausible, even under micro-aerobic conditions.

2.7.6.5 Other polymers

Proteins, chitin, cutin, waxes and suberin are persistent polymers that are available to a 
broad spectrum of fungi for an extended period (Cooke and Whipps 1993). These 
polymers tend to be minor components of plant litter in aquatic environments, so 
research into aquatic decomposition has tended not to focus on them. Storage polymers 
such as starch, inulin, glycogen and lipids are theoretically available to a wide range of 
fungi. In practice, they are only utilised by fungal parasites and the primary colonisers of 
dead material (Cooke and Whipps 1993).
2.8 Summary

While the body of knowledge concerning fungal ecology has established a firm understanding of fungal metabolism and taxonomy, little work has been carried out in aquatic environments within Australia, and while fungal specimens have been identified from standing waters in Australia, no ecological studies have taken place in floodplain wetlands. It is therefore necessary to explore fungal ecology in Australian floodplain wetlands from the most basic of starting points, and ask the question, “What role do fungi play in the ecology of ephemeral floodplain wetlands?” Fundamental aspects of this question are examined in Chapter 3, where the fungal community structure is compared between sediment and plant detritus substrates in a typical Australian floodplain wetland.
Chapter 3

Pilot Study: Fungal Community Structure on a variety of substrates from within a floodplain wetland.

3.1 Introduction

Research conducted on floodplains of the Rhône (Pattee and Chergui 1995) and Garonne Rivers (Baldy, Chauvet et al. 2002) has shown that aquatic fungal communities differ between rivers and lentic habitats on their floodplains. This has been attributed to differences in temperature, aeration (Dix and Webster 1995), dissolved organic carbon concentrations and flow (Pattee and Chergui 1995). However, large differences in community structure have also been observed between submerged wood and submerged leaves (Gulis 2001) and between different leaf species (Thomas, Chilvers et al. 1992). Differences have also been noted between similar substrates in different locations (Suberkropp and Chauvet 1995) and these differences have been attributed to differences in riparian vegetation and abiotic variables.

To date, there are no published studies that have investigated fungal community structure in Australian floodplain wetlands, although there have been numerous studies that have investigated the importance of bacteria in these systems (Boon 1990; Boon and Sorrell 1991; Boon, Virtue et al. 1996; Bunn and Boon 1993; Burns and Ryder 2001b; Ford, Boon et al. 2002). A number of taxonomic studies have recorded the occurrence of aquatic fungal species in rivers (Cowling and Waid 1963; Price and Talbot 1966; Thomas 1992; Thomas, Chilvers et al. 1989; Thomas, Chilvers et al. 1992) and non-flowing habitats (Hyde and Goh 1998), and fungal biomarkers have been isolated from biofilm growing on submerged wood (Scholz and Boon 1993a; Scholz and Boon 1993b).

Boon and his co-authors (1996) used phospho-lipid fatty acid (PLFA) profiles to investigate microbial diversity in floodplain wetland sediments. They were unable to detect fungal biomarkers and concluded that fungi were not important in wetland sediment carbon cycling. While many aquatic fungi species are tolerant of low oxygen environments (Field and Webster 1983; Medeiros, Pascoal et al. 2009b), and some are facultative anaerobes capable of fermentation (Reith, Drake et al. 2002), there is no evidence that fungi are able to dominate decomposer communities under anoxic
conditions. As the sediments of floodplain wetlands are known to become anoxic soon after inundation (Baldwin and Mitchell 2000; Mitsch and Gosselink 1993b), the findings of Boon et al. (1996) are plausible.

Plant detritus serves as a substrate for aquatic fungi in wetlands. Most of the allochthonous plant production in wetlands is not consumed by grazers (Brinson, Lugo et al. 1981), but is decomposed in the standing dead position or after it collapses to the sediment surface (Buesing and Gessner 2006). Allochthonous litter, derived from riparian vegetation, provides another possible substrate for fungal communities. In the aerial position, in the water column and on the surface of the sediments, conditions exist in which fungal communities might develop, although bacteria are known to dominate submerged detritus under anoxic conditions (O'Connell, Baldwin et al. 2000). Fungi dominate substrates such as these in rivers, particularly in the early stages of decomposition when particle size is large (Suberkropp 1991a).

While the activities of the decomposer community in the sediments of floodplain wetlands and fungal dynamics within wood biofilm have been investigated, there have been no reports of investigations into fungal dynamics on plant detritus in these systems. Indeed, the failure to detect fungal biomarkers has lead to the suggestion that fungi are not “a significant component of the microbial community” in floodplain wetlands (Boon, Virtue et al. 1996). Therefore it is first necessary to confirm the presence of fungi in Australian floodplain wetlands. Following this, the micro-habitats which fungi colonise should be determined. These microhabitats might be influenced by substrate type, location and water depth.

In the following experiment, T-RFLP analysis was used to firstly detect the presence of fungal DNA, then to compare fungal community structure profiles between wetland sediments, and allochthonous and autochthonous plant substrates, in a floodplain wetland. These profiles were then compared between sites within the wetland, and with sediment from the associated river. The community structure in sediments from a variety of water depths and submerged and standing dead litter were also compared. The results of this investigation were intended to generate hypotheses for further experiments.
3.2 Materials and Methods

3.2.1 Study site

Normans Lagoon is a billabong adjacent to the Murray River at Albury, NSW, Australia (146°56’06″E, 36°05’42″S; Figure 3.1). The billabong is 675m long and 40m wide, and 2-6 m deep along the central axis. Open water is interspersed with reed beds, and populations of the floating fern, *Azolla filiculoides* (Lam.) are often present. On the north-east side it is bounded by a road reserve, where the dominant riparian vegetation is *Eucalyptus camaldulensis* (Dehnh.) (Figure 3.2). Exotic tree species belonging to the genus *Salix* are also present. Stands of emergent macrophytes consisting of *Eleocharis sphacelata*, *Cyperus eragrostis* and *Juncus ingens* are present along the shore line. *Brasenia shreberi* is the dominant floating attached macrophyte with *Azolla filiculoides* also floating on the water surface. On the south-western side the lagoon is bordered by pasture. Water enters the lagoon from the Murray River at the downstream (west) end during minor flood events (18 000 ML/day), but fills from the upstream end during major floods (Gribben, Rees *et al.* 2003).

Figure 3.1: A map of Normans Lagoon, showing the position of sample sites. The inset shows the location of Albury-Wodonga, situated on the Murray River, which forms the border between the states of New South Wales and Victoria. Normans Lagoon is found on the north side of the Murray River, south-east of Albury, NSW.
Figure 3.2: The western site at Normans Lagoon. The riparian vegetation at this site is dominated by *Eucalyptus camaldulensis*. Outside this zone, the vegetation is influenced by rural land use.

3.2.2 Sample collection

Samples of sediment and plant material were collected from Normans Lagoon on October 20\textsuperscript{th}, 2006. Triplicate sediment samples were taken from the Murray River at Doctors Point, at the western end of the lagoon and from a site on the eastern side of the lagoon. Vegetation was sampled only from the two lagoon sites. Samples were collected in sterile specimen jars and frozen at -20°C on return to the laboratory, less than 1 hour later.

Sediment samples consisted of (a) dry soil sampled at a distance of 5 m from the water’s edge, (b) moist soils, sampled <1 m from the water’s edge, (c) recently submerged soil, sampled ~30cm into the lagoon from the water’s edge, (d) submerged flocculent material (floc) (from ~20cm water depth), possibly comprised of *Azolla filiculoides* detritus, (e) submerged sediment (from ~50cm water depth), and (f) submerged sediment from *Cyperus* sp. (east site) and *Juncus* sp. (west site) reed beds (20-50cm water depth).

Vegetation samples consisted of (a) *Eucalyptus camaldulensis* leaves from the sediment surface, (b) *Salix alba* leaves, yellowed and floating on the surface, (c) Standing dead and submerged *Juncus ingens* leaf blade, from the west site, (d) Standing dead and
submerged *Cyperus eragrostis* leaf blades, from the east site, and (e) submerged bark (species unknown).

### 3.2.3 Terminal restriction-fragment length polymorphism (T-RFLP)

DNA was extracted from sediment samples, using an UltraClean™ Soil DNA Isolation Kit (MoBio Laboratories, Inc, Solana Beach, CA, USA.) following the alternative protocol recommended for maximising yields. Optimisation of this method for use with both sediment and plant samples led to the following modifications: (a) The samples were centrifuged and the supernatant was removed, then weighed into bead tubes; (b) cells were lysed using a bead beater for 10 seconds on high, followed by 2 minutes on ice, and these steps were repeated three times, and (c) after adding solution S2, a protein precipitating reagent, tubes were incubated at 4°C for 1 hour rather than 5 minutes. The DNA extracted from these samples is hereafter referred to as the “genomic” DNA.

PCR was used to amplify the internal transcribed spacer region II (ITS2) of fungal ribosomal DNA (rDNA), found between 5.8S-coding sequence rDNA and Large sub-unit coding sequence (LSU) of the ribosomal operon (Martin and Rygiewicz 2005). The ITS regions (I and II) are more variable than the other subunits on the gene, and there are sequence variations among species (Kumar and Shukla 2005). Thus, this region of the fungal DNA is useful for developing community profiles.

The PCR reaction was carried out using Qiagen HotStarTaq® DNA polymerase (Quiagen, Hilden, Germany) and the accompanying reaction buffer. A 50 µL reaction volume was used, comprised of 0.5 µL Taq, 5 µL of 10x reaction buffer, 1 µL dNTP (dATP, dGTP, dTTP and dCTP, 10 mM of each; Genesearch, Arundel, Qld), 2 µL forward primer (0.4 µM per µL), 2 µL reverse primer (0.4 µM per µL), 1 µL genomic DNA, and 38.5 µL sterile milliQ water. Genomic DNA was used either undiluted or diluted to 10% in sterile milliQ water. The forward primer used was ITS3 (5'- TCG ATG AAG AAC GCA GC – 3') and the reverse primer was ITS4 (5'-TCC TCC GCT TAT TGA TAT GC – 3') (White, Bruns et al. 1990). Primers were synthesised by Gene Works (Hindmarsh, S.A.). The forward primer carried a fluorescent label (5, 6 - Carboxyfluorescein) at the 5' end. These primers produce a DNA fragment approximately 350 bp in length (length may vary between species) and are specific to fungi (Kumar and Shukla 2005).
Amplification was carried out in an iCycler (Bio-Rad, Hercules, CA, USA) thermocycler using a Touchdown protocol (Don, Cox et al. 1991). This approach has been used to amplify fungal DNA (Green, Freeman et al. 2004; Kasuga, Taylor et al. 1999; Meyer, Carta et al. 2005), and in conjunction with T-RFLP (Arteau, Labrie et al. 2010) previously. The PCR protocol consisted of initial denaturation at 95°C for 15 minutes (1 cycle), then 15 cycles of denaturation at 94°C for 15 seconds, annealing for 15 seconds, and extension at 72°C for 60 seconds. Annealing temperature was reduced by 1°C after each cycle, commencing at 60°C and ending at 45°C. A further 20 cycles then followed with denaturation at 94°C for 15 seconds, annealing at 48°C for 15 seconds, and extension at 72°C for 60 seconds. A final cycle was included with denaturation at 94°C for 15 seconds, annealing at 48°C for 60 seconds, and extension at 72°C for 6 minutes. The PCR product was then held at 4°C until removed from the thermocycler and stored at -20°C. PCR product was visualised under UV light on a 1% agarose gel incorporating ethidium bromide (0.4 µg.ml⁻¹). Retention of the amplicons was compared with a lambda digest size standard to determine their approximate length.

The PCR product contains primers, enzymes, salts and other substances that may become problematic during the restriction digest or subsequent electrophoresis. These contaminants were removed using the UltraClean PCR Clean-up Kit (MoBio Laboratories, Inc, Solana Beach, CA, USA), following the manufacturer’s instructions. However, when samples were combined with the Spinbind buffer the pH increased to above 8.5, so this was adjusted to less than 7.5 by adding 10 µL of 3M Sodium Acetate (molecular biology grade, Gene Works, Hindmarsh, S.A.).

Approximately 80-100 ng of fungal DNA was digested with 3 µL of Hin6I restriction enzyme (1 unit / µL) at 36 – 37°C for 3 hours, using a reaction volume of 25 µL. After this time, 75 µL of ice cold ethanol was added to each tube to halt the reaction. Then 2.5 µL of 5 M NaCl was added to each tube with gentle mixing and incubation overnight at -20°C to precipitate the DNA. The tubes were then centrifuged at 4°C for 30 minutes before the supernatant was removed. The DNA was dried before being dispatched for fragment analysis. Fragment analysis was performed at the Australian Genome Research Facility Ltd (AGRF) at The Walter and Eliza Hall Institute in Melbourne, Australia. The
3730 platform (Applied Biosystems, Carlsbad, CA, USA) capillary electrophoresis system was used.

Fragment size and peak area data for each sample were tabulated, and fragment sizes were rounded to the nearest whole number. Peaks less than 30 base pairs in length were disregarded, to exclude possible contributions from primers. The data for each sample were normalized, by converting each peak area to a percentage of the total peak area for that sample. The peak rejection threshold was determined by comparing the loss in data peaks when all data comprising less than 0.01, 0.1, 0.25, 0.5, 1 and 3% of the total sample data were removed. The threshold was set at the level where the loss of T-RFs per profile was minimal and sufficient T-RFs remained to analyse the community profile (Sait et al, 2003) (See Appendix 1). Data were normalised and a minimum threshold of 0.25% of total peak area was applied. This serves to remove bias stemming from differing amounts of PCR product being subjected to gel electrophoresis, while having a minor effect on the overall area of the remaining T-RF peaks (Rees, Baldwin et al. 2004; Sait, Galic et al. 2003).

Data were analysed with multi-dimensional scaling (MDS) using Primer 6 for Windows, version 6.1.6 (Primer-E Ltd, Plymouth, UK). The data were presence/absence transformed, and a Bray-Curtis resemblance matrix was then generated analysing between samples. This resemblance matrix was analysed using MDS with 10 restarts. Hierarchical Cluster analysis, in group average cluster mode, was used to generate clusters that were then overlaid on MDS plots. Analysis of Similarity (ANOSIM) (Clarke and Warwick 2001), was performed, and the global R statistic was calculated to determine the significance of observed separation between groups.

3.3 Results

The method of DNA extraction from wetland sediments and plant material proved successful in most instances. The use of the “touchdown” thermocycler program in PCR proved suitable for amplifying fungal DNA, and the majority of the product obtained was shown to be less than 500bp in length. This confirmed that the ITS2 section of the ribosomal DNA was amplified. Terminal restriction fragments of 97 different lengths were isolated. Fragments ranged in length from 35 to 510 bp.
3.3.1 Sediments and vegetation

The most important factor in structuring fungal communities in Normans Lagoon was substrate (Figure 3.3). At the 25% similarity level sediment samples and flocculent material from the sediment surface clustered into a group, while plant detritus formed a separate group. Sediment and plant substrates were dissimilar (ANOSIM: R=0.763; P<0.005), while floc and sediments were not distinguishable (pair-wise ANOSIM: R= -0.067; P=0.563). The presence of outlying samples indicated high variability in the structure of fungal communities. Fungal communities present on Eucalyptus sp. leaves were dissimilar to those on sediments (pair-wise ANOSIM: R=0.716; P<0.005) and floc (pair-wise ANOSIM: R=0.630; P= 0.100).

Figure 3.3: MDS plot of all T-RFLP data collected from Normans Lagoon. Sediment samples are shown in black, floc samples are shown in grey and plant detritus samples are shown in white. Symbols, as shown in the legend, have been used to differentiate between substrate types.

3.3.2 Site effects

Another factor influencing the structure of fungal communities was location, although the data indicated that location was a less important factor than substrate. Sediments from the Murray River at Doctors Point, and the east and west sites at Normans Lagoon formed clusters at approximately 30% significance level (Figure 3.4). There was a low level of separation between the east, west and Doctors Point sites (ANOSIM: R=0.101; P<0.05), indicating that variability between sites was only slightly larger than variability within sites. Differences between the east and west sites, the east site and Doctors Point, and the west site and Doctors Point (Pair-wise ANOSIM: R=0.233, P<0.05; R=0.514,
P<0.05; R=0.346, P=0.063, respectively) were minor, but showed a trend towards increasing difference with increasing distance between sites.

When sediment and floc samples were considered separately to plant substrates there was some low level separation between groups (ANOSIM: R= 0.302; P<0.05). Sediment samples taken from the Murray River at Doctors Point differed from samples in the east and west (Pair-wise ANOSIM: R=0.514, P<0.05; R=0.346; P=0.063, respectively) of the lagoon (circles overlaid on Figure 3.4). This indicated a possible difference between fungal community structure in river and wetland sediments.

![Figure 3.4: MDS plot of T-RFLP data from sediment and floc samples. Samples from the west lagoon site are shown in black, samples from the east lagoon site are shown in grey, and samples from the river at Doctors Point are shown in white. Symbols indicate sediment moisture class, i.e. dry (star), damp (diamond), water’s edge (squares), inundated (hexagons), deep (circles) and reed bed (triangles). Overlaid circles are intended to indicate site groups and have no statistical meaning.](image-url)

3.3.3 Moisture effects

Samples were classified according to a relative scale as either “dry”, “damp” or “wet”. Moisture content was not measured. All inundated samples were considered to be “wet”. “Dry” sediments were not inundated and had been exposed for some time. “Damp”
sediments had been inundated recently but were exposed to the air at the time of sampling. Some separation of sediment and floc samples according to moisture class was evident (ANOSIM: R=0.361; P<0.05), although differences between dry and damp, dry and wet, and damp and wet classes (Pair-wise ANOSIM: R=0.407, P=0.100; R=0.392, P<0.05; R=0.331; P<0.05, respectively) were small. Where plant and sediments from the west site were considered together, the level of separation remained low (ANOSIM: R=0.382, P<0.05). However, when sediments (only) from the west site were considered separately, moisture classes were found to have dissimilar fungal communities (ANOSIM: R=0.773; P<0.005) (Figure 3.5). Dry sediments differed from damp and wet sediments (Pair-wise ANOSIM: R=0.500, P=0.333; R=0.410, P=0.091, respectively) but the level of significance was low due to a small number of replicates for damp and dry sediments. This suggested that variation due to substrate and site were more important in determining the structure of fungal communities in Normans Lagoon than moisture class, but that moisture effects may have been important within sites and substrate types.

Figure 3.5: MDS plot of T-RFLP data from sediment and floc samples from the Normans Lagoon west site. Symbols indicate sediment moisture class, i.e. dry (stars), damp (diamonds), water’s edge (squares), inundated (hexagons), deep (circles), and reed bed (triangles). Dry samples are shown in white, damp samples in grey and wet sediments in black. Overlaid circles illustrate samples are grouped by sediment moisture content, but have no statistical meaning.
3.3.4  **Response of fungal community to substrate species differences**

Plant detritus samples showed little separation by site (ANOSIM: R=0.253, P=0.079), with the species of the plant substrate (excluding bark and willow due to a low number of replicates) appearing to influence fungal community structure more strongly (Figure 3.6). The fungal community structure differed between *Eucalyptus* sp., *Juncus* sp. and *Cyperus* sp. leaves as they decomposed in the wetland (ANOSIM: R = 0.573; P<0.005), but while fungal communities on eucalypt leaf samples were variable, they differed from *Cyperus* sp. (Pair-wise ANOSIM: R=0.741; P<0.05) but not from *Juncus* sp. (Pair-wise ANOSIM: R=0.250; P=0.200), while *Cyperus* sp. and *Juncus* sp. were dissimilar (R=0.661; P=0.067). Separation between detritus samples based on moisture status was not significant (R = -0.042, P = 0.509).

![Figure 3.6: MDS plot of T-RFLP data from plant detritus samples from Normans Lagoon. The west site is shown in black, while the east site is shown in grey. Overlaid circles indicate plant species groupings but have no statistical meaning.](image)

3.4  **Discussion**

It was possible to amplify the ITS2 region of the rDNA specific to fungal communities from sediments and plant detritus using PCR. This indicates that fungi are present in Normans Lagoon and are thus likely to be present in similar floodplain wetlands in
Australia. Analysis of terminal restriction fragment length from a range of substrates sampled from throughout the wetland and the adjacent river shows that the structure of these fungal communities is influenced by substrate composition, site and moisture class.

3.4.1 Changes in fungal community structure with time

The fungal community composition of submerged leaves is known to change over time (Canhoto and Graça 1996; Pattee and Chergui 1995; Suberkropp and Klug 1976). As all fungal substrates were collected from the wetland without knowledge of their total exposure time, exposure time is a possible source of variation in fungal community structure on plant detritus. Specifically, this effect may partly explain the high variability observed in the fungal community structure on Eucalyptus sp. leaves, which may float on the water’s surface for a period, before becoming submerged and possibly buried in sediments. Each of these changes is likely to influence the composition of the fungal community, and succession due to the changing composition of the substrate induces further changes in fungal community structure.

The composition of fungal communities on submerged plant litter may be strongly influenced by randomly arriving “founder” species that tend to maintain a presence in the community over time (Fischer, Bergfur et al. 2009; Pattee and Chergui 1995), even though their abundance may change. The presence/absence transform used gives the same level of importance to all species presence, regardless of abundance. These founder species may increase variability in fungal community structure between samples. The importance of these temporal factors can not be inferred using the current data set.

3.4.2 Fungal community structure differences in Normans Lagoon

Fungal communities found in surface flocculent material (floc), possibly associated with detritus from Azolla filiculoides, were not significantly different from sediment samples. This was not unexpected as floc samples may have contained some sediment. However, fungal community structure on plant detritus differed significantly from those on sediment and floc. These differences between fungal communities are likely to be associated with differing requirements for nutrition, different tolerances to sediment anoxia, and the ability to compete with other micro-organisms for resources.

Where plant detritus is decomposed in rivers, fungi dominate the decomposer community when particle size is large. When particle size is small, bacteria are the dominant
decomposers (Suberkropp and Klug 1976). Fungi have been shown to selectively colonise resources depending on their quantity, quality and distribution, and in a manner that minimises energy expenditure (Boddy 1993; Bolton and Boddy 1993). Plant detritus is a large, higher quality (more nutrients, less recalcitrant compounds), concentrated resource that the fungal mycelium can consume as it extends, while organic resources in the sediments are smaller, more dispersed, and lower quality. This suggests that aquatic fungi would be likely to colonise plant detritus in preference to sediments. As bacteria are not constrained within a multicellular form, they may be transported passively by flow to positions close to the resource and obtain energy from within the sediments with greater efficiency. Previous research investigating microbial dynamics in wetland sediments (Boon, Virtue et al. 1996) indicates that bacteria dominate decomposition.

Inundated wetland sediments are usually anoxic (Boon and Mitchell 1995), and while the absence of oxygen does not inhibit the metabolic activities of a range of anaerobic sediment bacteria, few aquatic fungi are able to survive these conditions in active life stages (Cooke and Whipps 1993). For these reasons we can expect the fungal community characteristic of sediments and floc in Normans Lagoon to be composed of species or life stages tolerant of low oxygen conditions and facultative anaerobes such as yeasts, chytrids and Oomycetes. DNA derived from dormant propagules of fungal forms inhabiting the water column or floodplain soils may also be represented by terminal restriction fragments (Lee and Taylor 1990). Indeed, it is possible that propagules comprise a large proportion of the sediment fungal community. If the active life stages of species producing these propagules are associated with Azolla filiculoides communities, this would explain similarities in fungal community structure between floc and sediments. Alternatively, the fungal community on floc samples may have been associated with sediment particles that comprised a proportion of the floc.

Fungal community structure on decaying leaves differs with leaf species (Canhoto and Graça 1996; Thomas 1992). In particular, the leaves of Eucalyptus species support different fungal assemblages to both deciduous European species (Canhoto and Graça 1996) and other Australian indigenous trees such as Acacia sp. (Thomas 1992; Thomas, Chilvers et al. 1992). The leaves of Eucalyptus species contain high levels of tannins and essential oils that have been shown to inhibit growth in aquatic hyphomycetes (Canhoto and Graça 1999) and cause a lag in fungal colonisation with respect to other tree species
(Graça, Pozo et al. 2002). For these reasons it is likely that *Eucalyptus* leaves support specialised fungal taxa (Thomas, Chilvers et al. 1992). However, all of the previous studies have examined fungal colonisation of *Eucalypt* sp. leaves in rivers, and it is possible that fungal colonisation patterns are different in wetlands. The limited results for these leaves from Normans lagoon show high variability between replicates, but indicate some similarity with other submerged plant detritus.

Sediment sample outliers consisted of dry and damp exposed or recently inundated sediments that may have contained plant roots or other plant material. These samples clustered with submerged *Eucalyptus* sp. leaves and bark, which are likely to be high in lignin (Cork and Pahl 1996). It is likely that these substrates supported a distinctive fungal community characterised by specialist fungal species capable of degrading refractory organic material such as lignin.

The lignin content of submerged decaying leaves is also known to influence the structure of the fungal community (Gulis 2001). Wood and bark are rich in lignin, while *Eucalyptus* sp. leaves have a relatively high lignin content when compared with the leaves of *Salix* sp., for example (Gessner and Chauvet 1994; Janssen and Walker 1999). The degradation of lignified plant tissues requires specialised enzymes (Deobald and Crawford 1992), so fungal species able to secrete these enzymes will have an advantage on lignin-rich plant detritus, and this advantage will influence community structure.

There is a weak trend shown for fungal community structure in sediments and floc to cluster according to location within the wetland, and in the river. This indicates some spatial heterogeneity within the wetland. Heterogeneity may be driven, in part, by differences in sediment texture. Visual examination suggested that sediments in the Murray River were sandy, while those at the western side of the lagoon were silty, and those in the eastern site were higher in clay. Sediment texture will influence gas diffusion and will thus influence dissolved oxygen concentrations. Sedimentation rate will affect the duration of substrate availability to aerobic decomposers.

While location influences fungal community structure it is not a simple variable, but is composed of spatial differences in a range of environmental variables. These variables might include vegetation, incident light, land use and prevailing winds, and their
interaction. Spatial differences in riparian and aquatic vegetation influence fungal community structure in terms of the availability of substrates, but as it is a persistent feature, it also influences the pool of available fungal propagules (Suberkropp 2001). Shading may influence the growth of algae, which would in turn influence the structure of fungal communities (Albariño, Villanueva et al. 2008). Land use that allows cattle to access the wetland may result in loss of vegetation, erosion and nutrient enrichment (Robertson, Klomp et al. 1997), which would in turn influence fungal community structure. Prevailing winds are of significance due to the abundance of the floating fern, Azolla filiculoides. This fern will tend to move with the wind and accumulate at the end of the wetland, providing an abundant, nitrogen rich substrate for fungi at the down wind end of the wetland. Thus, it is difficult to determine which aspects of a location are influencing fungal community structure.

The effects of moisture status are difficult to discern, as substrate and site effects tend to mask them. However, when these factors are controlled, some impacts of moisture status are discernable. Firstly, dry and damp soil fungal community structures differ from community structure in inundated sediments, although the depth of overlying water does not appear to be important (although samples were collected from water depths of less than 0.5m). This may indicate that the fungal community differs between moisture limited terrestrial conditions and aquatic conditions, which are likely to be limited by oxygen availability (Gribben, Rees et al. 2003). Damp soils, and sediments associated with macrophyte stands, represent positions on a gradient between these two community types. An oxygen availability gradient is further supported by small differences in fungal community structure between standing dead and inundated reed (Cyperus and Juncus sp.) detritus. The willow leaf (Salix sp.) was found floating on the surface of the wetland, and may support a fungal community adapted to aerial conditions, hence its affinity with standing dead reeds.

3.4.3 The ecological significance of these findings

The data presented here suggest that fungal community structure is influenced by substrate, site factors and moisture status. This implies that fungal community diversity is strongly dependent upon the diversity of the plant detritus available, and is vulnerable to the environmental forces that threaten plant diversity. There are indications that elements of the sediment fungal community are independent of those found on plant detritus, and
these elements may include propagules and vegetative forms of species that inhabit the water column, parasitising algae and other organisms, or decomposing dead biota from the water column as it encounters the sediment surface. Thus, the sediments may contain a “seed bed” of fungal propagules that remain dormant during unfavourable conditions, such as anoxia or desiccation, and are able to colonise suitable substrates when conditions are more favourable. While this is a positive attribute in terms of leaf conditioning and decomposition, it may have negative aspects associated with the prevalence of parasitic and pathogenic fungal species.

3.5 Conclusions

It is clear from the data presented in this chapter that fungi were present in all of the substrates examined from Normans Lagoon, and strong differences were seen between the fungal community structures in the sediments and on plant detritus. While lack of replication in this pilot study gives me reason to defer drawing further conclusions, the data suggest that factors such as the species of plant material, site related variables and the moisture status of sediments may be important structuring factors for fungal communities in floodplain wetlands.

We have seen that the effects of substrate, moisture treatment and site interact to produce variation in fungal community structure. It is also likely that community structure changes with time. In chapters to follow, I will explore these effects further. In Chapter 4, I present a laboratory experiment in which I compare fungal community structure on two leaf types between terrestrial and aquatic conditions, and follow these changes over time. In addition, the abundance and activity of these fungi are measured. In Chapter 5, I examine the relationship between sediment moisture content and fungal community structure in the sediments of a floodplain wetland, and measure the abundance of these fungi. In Chapter 6, fungal community structure and other fungal dynamics on submerged *E. camaldulensis* leaves are compared between rivers and wetlands at three locations.
**Fungal activity on Eucalyptus camaldulensis leaves under aquatic conditions is enhanced by terrestrial aging.**

### 4.1 Introduction

In Chapter 3 I showed that fungi present in Australian floodplain wetlands are colonising a variety of plant substrates as well as sediments. In this chapter I further explore the activities of these fungi by examining how the biomass and activity of the fungal community changes over time, and how this affects the decomposition of the substrate. A laboratory experiment was used to examine fungal dynamics on fresh and terrestrially aged leaves of the river red gum (*Eucalyptus camaldulensis*, Dehn. Myrtaceae) under moisture conditions designed to simulate those on the floodplain and in a floodplain wetland.

*E. camaldulensis* is the dominant tree species in floodplain ecosystems throughout the Murray-Darling Basin, and is known to be important in aquatic food webs (Reid, Quinn *et al.* 2008) and carbon cycles (Baldwin 1999; Francis and Sheldon 2002; O'Connell, Baldwin *et al.* 2000; Robertson, Bunn *et al.* 1999). Under natural flow regimes, these wetlands flood predominantly in winter / spring, and summer / autumn flooding was rare prior to river regulation (Bren 1988). As *E. camaldulensis* leaves fall predominantly in summer (Glazebrook and Robertson 1999), abscised leaves lie on the dry floodplain for some months prior to inundation, undergoing terrestrial aging. River regulation has lead to more frequent flooding of wetlands in summer (Bren 1988; Maheshwari, Walker *et al.* 1995), resulting in more frequent inundation of freshly abscised leaves.

Fungal dynamics on leaves are strongly influenced by the chemical composition of the substrate (Canhoto and Graça 1996). Fresh eucalypt leaves have been considered to be poor fungal substrates due to high contents of oils, tannins and polyphenols (Canhoto and Graça 1999; Graça, Pozo *et al.* 2002). However, when eucalypt leaves spend time on the floodplain as part of the litter layer, they are exposed to leaching and ultra violet radiation, and may be colonised by microbes or grazed upon by fauna. This alters their chemical composition (Baldwin 1999), and is likely to alter their quality as a fungal substrate.
Fungal dynamics have never been examined in Australian floodplain wetlands. A few studies have examined the aquatic decomposition of plant litter under lentic conditions, without specifically examining the activity of fungi. These studies suggest more rapid decomposition of leaves under submerged conditions (Glazebrook and Robertson 1999) and invertebrate activity (Briggs and Maher 1983). Differences in decomposition rates in wetlands were attributed to leaf composition (Janssen and Walker 1999), the presence of plant-derived oils (Bailey, Watkins et al. 2003), season of inundation (Watkins, Quinn et al. 2010) and nutrient dynamics (Shilla, Aseada et al. 2006). Studies examining the fungal contribution to leaf decomposition rates in wetlands in other countries have shown that rates are not influenced by differing flooding and drying frequencies (Inkley, Wissinger et al. 2008), and are similar in the main channel and associated floodplain wetlands (Baldy, Chauvet et al. 2002). Anoxic conditions in standing waters and sediments are also known to slow or halt fungal activity (Medeiros, Pascoal et al. 2009a; O'Connell, Baldwin et al. 2000; Pattee and Chergui 1995).

Fungal community structure on allochthonous litter can be expected to differ between the terrestrial conditions on the floodplain and aquatic conditions within the wetlands (Pattee and Chergui 1995). While terrestrial Eucalyptus sp. leaf litter is colonised by fungi such as Mucor sp., Rhizopus sp., Aspergillus sp., Penicillium sp., Trichoderma sp., Alternaria sp., Cladosporium sp. and Fusarium sp. (Eicker 1973), leaves submerged in still water are more likely to be colonised by aquatic hyphomycetes e.g. Alatospora sp., Anguillospora sp., Clavariopsis sp., Flagellospora sp., Lunulospora sp., Tetrachaetum sp., Tricladium sp. (Thomas, Chilvers et al. 1989; Thomas, Chilvers et al. 1992), aero-aquatic hyphomycetes and stramenopiles (Pattee and Chergui 1995; Voglmayr 1997). The transition from a terrestrial assemblage to an aquatic assemblage has not been investigated on the leaves of Eucalyptus species.

Differences in species composition on leaves in terrestrial and aquatic environments are likely to result in differences in total fungal biomass and losses of mass and carbon from decomposing leaves, as a result of competitive interactions (Mille-Lindblom, Fischer et al. 2006a) and differing enzymatic capabilities (Abdel-Raheem and Shearer 2002; Abdullah and Taj-Aldeen 1989; Valikhanov 1988). As fungal colonisation occurs in two phases (Verma, Robarts et al. 2003), we can expect fungal dynamics to reflect exploitation of starches and pectins by early colonisers, and the use of cellulose and
lignin one to two months later by secondary colonisers (Dix and Webster 1995; Pattee and Chergui 1995; Sinsabaugh, Moorhead et al. 1994). However, this pattern of activity will be influenced by the availability of moisture and oxygen, and by the chemical properties of the fungal substrate.

Here, I test the hypothesis that fungal community structure, biomass accumulation and extra-cellular enzyme activity differ between fresh and terrestrially aged E. camaldulensis leaves, and that these differences influence mass and organic matter loss from the leaves. I further hypothesise that fungal community dynamics differ between terrestrial and aquatic conditions and with incubation time. Freshly abscised and terrestrially aged E. camaldulensis leaves were placed in mesocosms simulating dry and moist terrestrial conditions and aquatic conditions, and fungal dynamics were compared between moisture treatments, substrate and over time.

4.2 Methods

4.2.1 Mesocosms

_Fresh_ and _aged_ leaves were obtained in May 2007 from the floodplain at Normans Lagoon, Albury, N.S.W (146°56’06”E, 36°05’42”S). This site is described in Chapter 3. Whole leaves were selected, and those damaged due to insect activity were not excluded. Leaves removed from the litter layer surface were considered to be aged if they no longer had any green colouration. _Fresh_ leaves were removed from branches that had blown to the ground in a storm 3 days earlier (sensu Baldwin et al. 1999). In the laboratory, both _fresh_ and _aged_ leaves were stapled into packs consisting of three leaves, labelled with a square of water-proof paper, weighed (DM₀) and dried in a dehydration oven at 50°C for 2 weeks.

Wetland sediment and wetland water were also obtained from Normans Lagoon. Living vegetation was removed from sediments, which were then mixed until homogenous, and then oven dried at 30°C for 7 days. Water was filtered through muslin cloth to remove large particles and invertebrates.

Six mesocosms were assembled that consisted of 60 x 30 x 30 cm fish tanks containing 4L of homogenised wetland sediment spread evenly over the base to a depth of 2 cm (Figure 4.1). Sediments were covered in nylon mesh to prevent leaf burial. Leaf packs of
fresh and aged leaves were distributed randomly between moisture treatments. Weighted wire mesh was placed over the leaves in all treatments. This was intended to ensure leaves in the wet treatments were submerged. Mesocosms were kept at 20°C, under predominantly dark conditions. The tanks were loosely covered with aluminium foil to help to maintain moisture status without impeding air flow.

Treatments are described as dry, moist and wet. In the dry treatment, no water was added to these tanks over 140 days of the experiment. In the moist treatment, enough wetland water was added to the sediments so that they were saturated, but no water pooled at the surface. In the wet treatment sediments were saturated, and a water depth of approximately 15 cm was maintained by occasional addition of filtered wetland water. Wet tanks were aerated with a bubbler to prevent the onset of anoxia. Leaf packs were removed from the treatment tanks after 1, 2, 4, 8, 16, 32, 64, 100 and 140 days. Preservation methods were specific to the analytical method.

![Figure 4.1: A schematic diagram of mesocosm design. All mesocosms were comprised of fish tanks, with a 2cm layer of wetland sediment in the bottom. This was covered with nylon mesh, and leaf packs were placed upon the mesh. Leaf packs were covered with weighted wire. In wet treatments, filtered wetland water was added to a depth of 15cm.](image)
4.2.2 Leaf mass loss and loss on ignition

Dried leaf packs were weighed prior to placement in the tanks (DM\textsubscript{1} for Day 0 leaves). On each sampling occasion, 3 leaf packs for each treatment were transferred into pre-fired and pre-weighed crucibles. Wet leaves were gently rinsed under running tap water to remove sediment from the leaf surface. The wet weight (WM) was recorded prior to drying at 105°C for 48 hours. Crucibles were brought to room temperature in a desiccator then re-weighed to determine the dry mass (DM\textsubscript{1}) of the leaves. Following this, leaves were ashed at 550°C for 1 hour to determine the loss of mass on ignition (AFDM). Mass loss as a percentage of the original mass was calculated as \(\frac{(\text{DM}_0 - \text{DM}_1)}{\text{DM}_0} \times 100\). Loss on ignition (%), an estimate of organic matter content, was calculated as \(\frac{(\text{DM}_1 - \text{AFDM})}{\text{DM}_1} \times 100\).

Data for mass loss and change in organic matter content were analysed using two-way analysis of variance (ANOVA) with replication (3) for each sampling time. Leaf type (fresh or aged) and treatment (dry, moist or wet) were compared with mass loss / organic matter as the dependent variable and time was as a covariant, pair-wise for treatments (Bärlocher 2005). Two-tailed t-tests (\(\alpha=0.05\)) were used to test differences between treatments and leaf type. Data analyses were performed using SPSS Statistics 17.0 (IBM, Chicago, USA). The slope of the mass loss curve (m) was also determined as a means of comparing mass loss rate between leaf types.

4.2.3 Fungal biomass

Ergosterol content of leaves was determined using the method published by Gessner (2005). Triplicate samples from all times were analysed for fungal biomass. Briefly, leaf samples were stored in HPLC grade methanol (Merck, Darmstadt, Germany), in the dark and at room temperature prior to analysis. A 12mm diameter disc was cut from each of three leaves, weighed and pooled for analysis. An additional set of three leaf discs was cut from the same leaves to determine the ratio of wet mass to dry mass and AFDM. The lipid fraction was extracted by refluxing in alkaline methanol for 30 min at 80°C. Extracts were filtered through 25mm diameter GF/C filters before purification with SEP-Pak solid phase extraction cartridges (Vac RC, tC18, 500mg of sorbent, Waters, Milford, MA, USA).
Ergosterol was quantified using a Waters HPLC system equipped with in-line degasser (AF), 717 plus auto-sampler, 600 pump controller and 2996 Photo diode Array detector (Waters, Milford, MA, USA). A 250mm x 4.6mm LiChrospher 100 RP-18 5u column (Grace Davison Discovery Sciences, Deerfield, IL, USA) was maintained at 33°C. The mobile phase was 100% HPLC grade methanol, on an isocratic gradient with a flow rate of 1.4 ml/min. Samples were dissolved in 100% HPLC grade propan-2-ol and 10µL was injected onto the column, with a run time of 20 minutes. The ergosterol peak was detected at 282nm after 12.060 minutes with a retention time window of 0.605 minutes. Data was processed using Empower™ software (Waters, Milford, MA, USA). Peak area was integrated using a traditional algorithm, with a minimum peak height of 420 au, minimum peak area of 4930 and a rejection threshold of 50.

Peak area was converted to ergosterol concentration based on comparison with a standard curve. Ergosterol content was then converted to fungal biomass using an estimated conversion factor of 5.5mg ergosterol per gram fungal biomass (Gessner and Chauvet 1993) and expressed as mg of fungal biomass per cm² leaf area. Fungal biomass data were analysed using 3-way ANOVA on the SPSS software package (IGM, Chicago, USA), analysing between leaf type, treatments and times (α=0.05). Paired sample t-tests were also carried out in SPSS.

4.2.4 Extracellular enzyme activity

4.2.4.1 Hydrolytic enzyme activity

Leaf samples were preserved by freezing at -20°C. Three leaves per sample were homogenised in 50ml of 50mM Na₂HCO₃ (pH 8.0), diluted in 1:1 with 50mM Na₂HCO₃, and then incubated on a shaker table for 30 minutes at 20 °C. Three replicate 150 µl aliquots of samples and a boiled control were combined 1:1 with substrate in a 96 well microtitre plate. Four different 4-methylumbelliferone (MUB) substrates (Sigma, St Louis, MO, USA) were used at a final concentration of 500 µM (Hendel and Marxen, in Graça et al., 2005). These were MUB-β-D-glucoside, MUB-α-D-glucoside, MUB-β-D-xyloside and MUB-β-D-cellobioside.

Fluorescence (Ex:Em = 355nm:460nm), was measured at 20°C using a Fluoroskan II plate reading fluorimeter (Labsystems, Finland), and recorded every 10 minutes at 0.5 sec/well for a 3 hour incubation period. Fluorescence was converted to nmol MUB using
a standard and standards + quench curve. The rate of increase in MUB concentration for each well was determined using linear regression. The rate of change of the boiled control was then subtracted from the mean activity rate to give the enzyme activity.

4.2.4.2 Oxidative enzyme activity

Phenol oxidase and peroxidase activity was measured using L-3,4-dihydroxyphenylalanine (L-DOPA) (≥ 99%, Fluka Cat no. 37830, Sigma-Aldrich, Steinheim, Germany) as the electron donating substrate (Hendel, Sinsabaugh et al. 2005). Leaf samples were preserved by freezing at -20°C. Three thawed leaves per sample were homogenised in 50 ml of MilliQ Water, diluted 1:10 in 50mM NaCH₃COO (pH 5.0) (acetate buffer), and then incubated on a shaker table for 30 minutes at 20°C. Samples were filtered through a GF/C filter prior to measuring absorbance. For phenoloxidase activity, 2 ml of filtered homogenate was combined with 2ml of 5mM L-DOPA. Four analytical replicates and 2 blanks were used. Blanks consisted of 2ml of filtered sample with 2 ml of acetate buffer. The assay for peroxidase was similar to the phenoloxidase, except that 0.2ml of 0.3% hydrogen peroxide was also added. Peroxidase controls contained 2 ml of filtered sample, 2 ml of acetate buffer and 0.2 ml of 0.3% hydrogen peroxide. The samples were incubated in the dark for 120 minutes on a shaker table at 20°C. Absorbance of samples was read at 460nm on a Cary spectrophotometer (Varian, Palo Alto, CA, USA). Activity was calculated according to Hendel et al., (2005).

Data for the activity of hydrolytic and oxidative enzymes were compared between treatments and substrate types, and over time using the t-Test for paired samples for means. Pearson’s R was used to compare fresh and aged leaves in each treatment, and for each enzyme, over days 0-16 and days 64-140. The activity of all enzymes, in all treatments at all times was analysed using multivariate 2-way crossed ANOSIM analysis on a 2-stage correlation matrix (Clarke, Somerfield et al. 2006) to determine whether consistent patterns were present in overall enzyme activity (Primer 6 for Windows, version 6.1.6, Primer-E Ltd, Plymouth, UK). The activity of individual enzymes was then compared between treatment, leaf type and times ($\alpha=0.05$) using the SPSS software package (IBM, Chicago, USA) to perform univariate 3-way ANOVA on normalised data.

4.2.4.3 Fungal community structure
Fungal community structure was assessed for samples from 0, 8 and 100 days incubation, using T-RFLP (see Chapter 3.2.3). After removal from the mesocosms, leaf packs were stored at -20°C in 70 ml sterile jars. Three 5mm diameter leaf discs were cut from each of three leaves in the leaf pack and frozen at -80°C overnight. Leaf discs were ground in a 2ml safe-lock tubes (Eppendorf AG, Hamburg, Germany) using a sterile mini-pestle, centrifuged for 1 minute at 10 x g and any supernatant removed. DNA was then extracted from the leaf material using the “UltraClean Soil DNA Isolation Kit” (MoBio Laboratories, Inc. California, USA) and the “Alternative Protocol (For maximum yields)” (Mo Bio Laboratories 2003). Extracted DNA was stored at -20°C prior to amplification. Some DNA extracts retained colour derived from the leaf substrate, which would interfere with amplification. These samples were further purified using the potassium acetate purification method (McVeigh, Munro et al. 1996). Polymerase Chain Reaction (PCR) was used to amplify the internal transcribed spacer region II (ITS2) of fungal ribosomal DNA (rDNA), found between 5.8S-coding sequence rDNA and Large sub-unit coding sequence (LSU) of the ribosomal operon (Martin and Rygiewicz 2005).

The PCR reaction conditions, PCR product clean-up, restriction digest, fragment analysis and data analyses were as described in Chapter 3.2.3.

4.3 Results

4.3.1 Leaf mass loss and loss on ignition

4.3.1.1 Mass loss from leaves

Mass loss from fresh and aged leaves in the dry, moist and wet treatments over 140 days is shown in Figure 4.2. Moist aged leaves at day 140 exhibited a gain in mass (not shown), with a corresponding large decrease in percentage organic matter. This is consistent with contamination of these samples with sediment, and therefore these data points were excluded from calculations. Moist fresh leaves showed a small mass loss on the same day, but as a decrease in the proportion of organic matter in the leaf was not observed these data points were not excluded.

Mass loss differed significantly between fresh and aged leaves, and between treatments, and a significant interaction between leaf type and treatment was detected (ANOVA: P<0.005). No significant mass loss was observed in the dry treatment (Figure 4.2, (i)).
the **moist** treatment (Figure 4.2, (ii)) mass loss differed between *fresh* and *aged* leaves. The maximum mass loss was seen in the **wet** treatment (Figure 4.2, (iii)).

*Aged* leaves showed no significant mass loss in the **moist** treatment (Figure 4.2, (ii)), while *fresh* leaves lost 23% of their initial dry mass after 100 days (t-test: $P<0.05$). In the **wet** treatment (Figure 4.2, (iii)) the most significant differences between leaf types occurred during the first week of incubation. Over this time, *fresh* and *aged* leaves lost 25% and 10% of their initial dry mass, respectively (t-test: $P<0.005$). Over the remaining decomposition time the rate of mass loss was higher for *aged* leaves ($m=0.09$ for *fresh* leaves; $m=0.18$ for *aged* leaves).

### 4.3.1.2 Mass loss on ignition

When considering all sample data together, the percentage of organic matter (Figure 4.3) differed significantly between treatments (ANOVA: $P<0.005$). When comparing between treatments, changes in organic matter content over time were not significantly different between **dry** and **moist**, but **wet** was significantly different to the other treatments (t-test: $P<0.05$), due to larger total losses of organic matter.

When considering all sample data together differences were observed between *fresh* and *aged* leaves, but these were not significant (ANOVA: $P=0.051$). When data for each treatment was analysed individually, a significant difference between leaf types was seen in **dry** and **wet** treatments (t-test: $P<0.05$).

### 4.3.2 Fungal biomass

The concentration of ergosterol in the leaves indicated that fungal biomass was higher on *aged* leaves than on *fresh* leaves (3-way ANOVA: $P<0.05$), and differences between treatments were significant (3-way ANOVA: $P<0.005$). While in the **dry** treatment biomass on *fresh* and *aged* leaves was always significantly different (t-test: $P<0.005$), in the other treatments differences decreased over time (Figure 4.4). **Wet** *fresh* leaves differed from **wet** *aged* leaves prior to 64 days incubation (t-test: $P<0.005$) as fungal biomass was very low on *fresh* leaves. After 64 days, this difference was not observed. In the **moist** treatment biomass differed prior to 16 days (t-test: $P<0.005$), but no difference was observed after that time.
*Fresh* leaves carried no fungal biomass on day 0, and very little subsequently accumulated on *fresh* leaves in the *dry* treatment. In the *moist* treatment, fungal biomass on *fresh* leaves increased until maxima were observed between 32 and 100 days, and then declined. *Wet* *fresh* leaves had low fungal biomass prior to day 64. Maximum fungal biomass was observed at day 64, and biomass decreased thereafter.

Prior to incubation, *aged* leaves supported approximately 3.5 mg.cm$^{-2}$ of fungal biomass. The temporal pattern of changes in fungal biomass on these leaves was similar across treatments. Biomass accumulated in two phases, with maxima at 2-4 and 32-64 days and minima at 16 and 140 days, but the amount of biomass at these points varied between treatments. Overall maximum fungal biomass occurred after 60 days in the *moist* treatment for both *fresh* and *aged* leaves.
Figure 4.2: Mass loss in the dry (i), moist (ii), and wet (iii) treatments over 140 days. Fresh leaves are indicated by closed symbols while aged leaves are indicated by open symbols. Mass loss, on the y-
axis, is shown as percentage of the mass of the leaf pack prior to incubation. Error bars indicate the standard error of the data point.

Figure 4.3: Changes in organic matter content of leaf packs over 140 days are shown for the dry (i), moist (ii), and wet (iii) treatments. Fresh leaves are shown as closed symbols while aged leaves are...
shown as open symbols. Organic matter content (y-axis) is expressed as a percentage of the leaf dry mass. Error bars indicate the standard error of the data point.
Figure 4.4: Fungal biomass (mg cm\(^{-2}\)) on eucalypt leaves, as calculated from ergosterol concentration. Differences in fungal biomass between aged (open symbols) and fresh (closed
symbols) leaves are shown for the dry (i), moist (ii) and wet (iii) treatments. Error bars indicate the standard error of the data point.

4.3.3  Enzyme activity

4.3.3.1  Combined enzyme activity

Multivariate analysis of combined hydrolytic and oxidative enzyme activity data indicated differences between fresh and aged leaves (ANOSIM: $R=0.346; P<0.05$; see Figure 4.5) and between treatments (ANOSIM: $R=0.337; P<0.05$). For individual enzymes, significant differences were found between treatments, leaf type, with time, and for all interaction affects (ANOVA: $P<0.05$).

4.3.3.2  Hydrolytic enzyme activity

Hydrolytic enzyme activity differed between fresh and aged leaves in the dry treatment (ANOSIM: $R=0.442; P<0.05$), but not in the moist (ANOSIM: $R=0.188; P<0.05$) or wet (ANOSIM: $R=0.162; P<0.05$) treatments. While activity on dry fresh and moist fresh leaves was generally close to zero, activity on dry aged and moist aged leaves was usually higher but decreased with time. Significant differences in activity over time on fresh ($R=0.4; P<0.05$) and aged leaves ($R=0.332; P<0.05$) were only observed in the wet treatment, where overall maximum activity was seen after 60 days.

The activity of cellobiohydrolase (Figure 4.6) and β-1,4-glycosidase (Figure 4.7) are indicative of the breakdown of cellulose. Cellobiohydrolase attacks the ends of cellulose chains removing cellobiose (a glucose dimer), while β-1,4-glycosidase attacks cellobiose to produce glucose monomers. Significant differences were observed between treatment, leaf type and time (ANOVA: $P<0.05$). The data show that significant activity towards cellulose was confined to the wet treatment (Figure 4.6 (iii)), although low activity was observed on aged leaves in the dry and moist treatments at earlier sampling times (Figure 4.6 (i) and (ii)). Activity towards cellobiose occurred on aged leaves in the dry and moist treatments (Figure 4.7 (i) and (ii)), with some activity occurring in fresh leaves after 100 days in the moist treatment. Strong activity towards cellobiose occurred in the wet treatment for both fresh and aged leaves (Figure 4.7 (iii)).
The activity of starch degrading enzymes is indicated by α-D-glucosidase (Figure 4.8), while activity of β-D-xylosidase indicates activity towards hemicelluloses (specifically xylans) (Figure 4.9). Leaf type, and leaf type x treatment significantly affected the activity of both of these enzymes (ANOVA P<0.05), while treatment and time x leaf type also significantly affected β-D-xylosidase activity. The strongest differences in the activity of these enzymes within a treatment occurred between moist fresh and moist aged leaves (ANOSIM: R=0.396; P<0.05). These data suggest that starch and hemicellulose breakdown was occurring on aged leaves in all treatments, with maximum degradation rates occurring in the dry treatment (Figure 4.8 and Figure 4.9, (i)). On fresh leaves, significant activity towards starch and hemicelluloses was only seen in the wet treatment (Figure 4.8 and Figure 4.9, (iii)), although some activity was recorded on fresh leaves in the moist treatment after 100 days (Figure 4.8 (ii) and Figure 4.9 (ii)).

4.3.3.3 Oxidative enzyme activity

The activity of phenol oxidase and peroxidase suggested that lignin breakdown was occurring. Oxidative enzyme activity peaked early in the incubation for all treatments and decreased with time (Figure 4.10 and 11). In the dry and moist treatments, higher activity was seen on aged leaves during this time. Phenol oxidase activity from day 60 onwards was low in the dry treatment, moderate in the moist treatment and higher in the wet treatment (Figure 4.10 (i), (ii) and (iii) respectively) and similar between leaf types. However, phenol oxidase activity on aged leaves after 100 days was lower than on fresh leaves for all moisture treatments. Similar levels of peroxidase activity were seen in the dry and moist treatments (Figure 4.11 (i) and (ii)), with activity being close to zero from day 60 onwards. Activity in the wet treatment (Figure 4.11, (iii)) continued at moderate levels from day 60 onwards, with lower activity observed for aged leaves after 100 days, than for fresh leaves. Maximum activity towards lignin was observed in the dry treatment at day 4 (Figure 4.10 and 11, (i)).

The activity of phenol oxidase and peroxidase (Figure 4.10 and 11) differed between leaf type and over time, with significant interaction effects (3-way ANOVA: P<0.005). There were also significant treatment effects for phenol oxidase activity (ANOVA: P<0.005).
The pattern of activity of oxidative enzymes in the wet treatment differed from the activity of hydrolytic enzymes. Where decreasing activity after 60 days was observed for all hydrolytic enzymes for both leaf types in this treatment, oxidative enzyme activity maintained moderate levels of activity, although activity on aged leaves was variable.

Figure 4.5: A three dimensional MDS plot based on a 2-stage resemblance matrix for oxidative and hydrolytic enzyme data for all times, treatments and leaf types. Fresh leaves are represented by solid symbols, while aged leaves are shown as open symbols. Treatments are symbolised by stars (dry treatment), diamonds (moist treatment) and circles (wet treatment). Arrows indicate that the direction of change in enzyme activity with different treatments differs between fresh leaves (solid arrow) and aged leaves (dashed arrow). Fresh and aged leaves in the wet treatment have similar enzyme activity.
Figure 4.6: The activity of cellobiohydrolase on aged (open symbols) and fresh (solid symbols) *E. camaldulensis* leaves in the dry (i), moist (ii) and wet (iii) treatments. Incubation time is indicated in...
days on the x-axis, while enzyme activity rate (μmol substrate converted per hour, per gram leaf dry weight) is shown on the y-axis. Error bars indicate the standard error of the data point.
Figure 4.7: The activity of β-D-glucosidase on aged (open symbols) and fresh (solid symbols) *E. camaldulensis* leaves in the dry (i), moist (ii) and wet (iii) treatments. Incubation time is indicated in days on the x-axis, while enzyme activity rate (µmol substrate converted per hour, per gram leaf dry weight) is shown on the y-axis. Error bars indicate the standard error of the data point.
Figure 4.8: The activity of α-D-glucosidase on aged (open symbols) and fresh (solid symbols) E. camaldulensis leaves in the dry (i), moist (ii) and wet (iii) treatments. Incubation time is indicated in days.
days on the x-axis, while enzyme activity rate (µmol substrate converted per hour, per gram leaf dry weight) is shown on the y-axis. Error bars indicate the standard error of the data point.
Figure 4.9: The activity of β-D-xylosidase on aged (open symbols) and fresh (solid symbols) *E. camaldulensis* leaves in the dry (i), moist (ii) and wet (iii) treatments. Incubation time is indicated in days on the x-axis, while enzyme activity rate (μmol substrate converted per hour, per gram leaf dry weight) is shown on the y-axis. Error bars indicate the standard error of the data point.
(i) Dry

Aged leaves
Fresh Leaves

(ii) Moist

Aged Leaves
Fresh Leaves

(iii) Wet

Aged Leaves
Fresh Leaves

μmol dopachrome.g⁻¹.leaf.hr⁻¹

Time (days)
Figure 4.10: The activity of phenol oxidase on aged (open symbols) and fresh (solid symbols) *E. camaldulensis* leaves in the dry (i), moist (ii) and wet (iii) treatments. Incubation time is indicated in days on the x-axis, while enzyme activity rate (µmol substrate converted per hour, per gram leaf dry weight) is shown on the y-axis. Error bars indicate the standard error of the data point.
Figure 4.11: The activity of peroxidase on aged (open symbols) and fresh (solid symbols) *E. camaldulensis* leaves in the dry (i), moist (ii) and wet (iii) treatments. Incubation time is indicated in
days on the x-axis, while enzyme activity rate (µmol substrate converted per hour, per gram leaf dry weight) is shown on the y-axis. Error bars indicate the standard error of the data point.

4.3.4 Community structure

Multivariate analysis of T-RFLP results (Figure 4.12) indicated that fresh and aged leaves carried different fungal communities prior to incubation (ANOSIM: R =0.937; P<0.005), but changes in community structure over time varied with treatment. In the dry treatment, no change was observed in either the fresh or aged community over 100 days (R = 1; P<0.05) (Figure 4.13).

Figure 4.13, (i)). In the moist treatment, fungal communities on fresh and aged leaves became more similar over time, such that over 100 days global R was reduced from 0.937 to 0.37 (P=0.20) (Figure 4.13, (ii)). The community present after 100 days was dissimilar to that found on both fresh and aged leaves prior to incubation (R = 0.512; P<0.005). Fungal community structure also changed over time in the wet treatment (Figure 4.13Figure 4.13, (iii)), however, in this case we observe the development of a new community that did not differ between fresh and aged leaves (R = 0.259; P=0.30), but was dissimilar to both communities present before incubation began (R = 0.993; P<0.005).

4.3.4.1 Community responses to moisture treatments

The pattern of change in fungal community structure after 100 days varied between treatments (ANOSIM: R=0.779; P<0.005). Fresh and aged samples at day 100 differed
in the **dry** treatment (Pairwise ANOSIM: $R=1$; $P=0.1$), but not in the **wet** (Pairwise ANOSIM: $R=0.259$) or **moist** (Pairwise ANOSIM: $R=0.370$) treatments (Figure 4.14).

![MDS plot of T-RFLP data](image)

**Figure 4.12**: An MDS plot of T-RFLP data from fresh (closed symbols) and aged (open symbols) *E. camaldulensis* leaves prior to incubation indicates that different fungal communities are present on the two leaf types (2D stress is 0.11). Circles are added to clearly define different groups shown in ANOSIM analysis.
Figure 4.13: MDS plots of T-RFLP data showing changes in fungal community structure in the dry (i), moist (ii), and wet (iii) treatments. Closed symbols represent fresh leaves while open symbols represent aged leaves. Incubation time is indicated by symbols. Pre-incubation samples are shown as circles, squares indicate 8 days incubation, the inverted triangle indicates 16 days incubation and upright triangles indicate 100 days incubation.
4.4 Discussion

This experiment has shown that fungal dynamics differ between fresh and aged *E. camaldulensis* leaves. While losses of mass and organic matter generally differed little between fresh and aged leaves, submerged fresh leaves lost more mass possibly as a result of leaching. Fungal biomass accumulation was generally delayed on fresh leaves, and total biomass was lower than that on aged leaves. Hydrolytic enzyme activity on fresh leaves was lower than that on aged leaves under terrestrial conditions, but not under aquatic conditions. Prior to incubation, fungal community structure differed substantially between fresh and aged leaves, and subsequent changes in community composition differed between treatments.

Fungal dynamics exhibited several important differences in response to moisture treatments. In the dry treatment, fungal community structure did not change, leaves lost little mass or organic matter, and fungal biomass and hydrolytic enzyme activity on fresh leaves were close to zero. On aged leaves fungal biomass and enzyme activity were higher, but did not generally change over time. Where the sediments were moist, some mass loss was observed in fresh leaves, fungal biomass was able to accumulate on both leaf types, hydrolytic enzyme activity towards plant structural carbohydrates was higher in aged leaves and the fungal community structure became more similar between leaf
types over time. When leaves were inundated, losses of mass and organic matter were significantly larger than in other treatments, fungal biomass accumulation was retarded on fresh leaves with respect to aged leaves, and enzyme activity was substantial and similar on both leaf types. Changes in fungal community structure in this treatment suggested that a transition from terrestrial to aquatic species had occurred.

Fungal colonisation of leaves is spatially variable, as fungi tend to extend in a radial pattern from a random point of contact (Chamier, Dixon et al. 1984), leading to high spatial variability in fungal biomass both within a single leaf and between leaves. This phenomenon has resulted in high standard deviations in metrics of fungal mass and activity.

4.4.1 Delays in fungal colonisation of “fresh” leaves

Previous studies of fungal species richness have shown that E. globulus leaves submerged in a stream are colonised by fungi 2 weeks after chestnut and alder leaves (Canhoto and Graça 1996; Chauvet, Fabre et al. 1997), and only 2 fungal species were present prior to 42 days in data presented by Canhoto and Graça (Canhoto and Graça 1996). The results presented here show that fungal colonisation of fresh E. camaldulensis leaves in standing water was delayed by at least 32 days after immersion. This lag time in fungal biomass accumulation on fresh leaves may be due to the presence of chemical substances that deter fungi. Oils, waxes and polyphenols such as those found in Eucalyptus sp. leaves protect plants against fungal colonisation (Boon and Johnstone 1997; Campbell and Fuchshuber 1995; Graça, Pozo et al. 2002) and grazing insects (Edwards 1982; Stone and Bacon 1994). Terrestrial micro-organisms and insects have adapted to overcome these defences (Ohmart and Edwards 1991), and in many cases insect grazing causes leaf wounds that facilitate fungal colonisation. Results from the moist treatment, which simulates the terrestrial floodplain environment, showed that fungal colonisers are able to overcome the effect of inhibitory substances in fresh leaves and accumulate biomass. However, data from the wet treatment suggest that aquatic organisms have not developed these tolerances.

The bulk of soluble polyphenols can be removed from submerged Eucalyptus leaves within 4 days of inundation (Baldwin 1999; Love 2005). If these polyphenols were the main cause of low fungal activity, the colonisation of fresh leaves by fungi could
theoretically be quicker under aquatic conditions than under terrestrial conditions. However, a lag in biomass accumulation suggests that many of the inhibitory substances in the leaves are poorly soluble in water (e.g. waxes and oils). This is consistent with the findings of Canhoto and Graça (1999), who reported that essential oils derived from *Eucalyptus* species were more inhibitory to aquatic fungi than tannins.

This study shows that while fungal biomass accumulation is inhibited in the weeks following the inundation of fresh leaves, hydrolytic enzyme activity occurs at similar levels in both fresh and aged leaves, throughout most of the incubation period. However, at day 16, hydrolytic enzyme activity was significantly lower on fresh leaves, suggesting that enzymatic break down of starch, cellulose and hemicellulose might also be inhibited soon after inundation (analysis of the changes occurring in substrate composition would be required to confirm this). As enzyme activity may be repressed in the presence of reaction end-products, enzymatic cleavage of structural polysaccharides may be inhibited by the presence of free sugars (Baldrian and Valaskova 2008; Chróst 1990). However, this is not likely to be the cause of inhibition in this instance, as free sugars would be utilised quickly by bacteria (Kaplan and Newbold 2003). In fact, breakdown is likely to be accelerated by the presence of bacteria that “mop-up” free sugars. External enzyme inhibitors such as eucalypt oils and tannins are a more likely cause of low activity. After 64 days incubation, the fungal community on fresh leaves was able to achieve the same level of biomass and activity as that seen on aged leaves. At this time the community composition was similar on the two leaf types. This suggests that substances inhibiting colonisation by aquatic fungi are removed over this period or their efficacy decreases with time.

The static conditions and evaporative concentration in wetlands may extend the period in which fungal growth and enzyme activity are inhibited by eucalypt leaf leachates, as compared to flowing systems. However, studies investigating the effects of *Melaleuca alternifolia* oil on wetland bacteria have found these effects to be small (Bailey, Watkins *et al.* 2003; Boon and Johnstone 1997). Increases in dissolved organic carbon due to the presence of eucalypt leachates may stimulate the activity of bacteria in the water column, causing decreases in the dissolved oxygen concentration (Howitt, Baldwin *et al.* 2007). Fungi are generally more sensitive to low oxygen concentrations than bacteria, and have a demonstrated susceptibility to eucalypt oils (Canhoto, Bärlocher *et al.* 2002; Terzi,
Morcia et al. (2007), so it is likely that these leachates would impact more profoundly upon fungal decomposers than bacteria.

4.4.2 Fungal activity under dry conditions

Birk (1979) found that mass loss from eucalypt leaves in the litter layer was largely due to mechanical fragmentation and insect consumption, with leaching and microbial activity being relatively insignificant. Conditions in the litter layer are generally moisture limited (O’Connell and Grove 1987), and results from the dry mesocosm are in support of these findings, showing that fungal communities on dry E. camaldulensis leaves did not grow or break down the leaf substrate over a 140 day period. It is possible that fungal decomposers are not important in the breakdown of terrestrial litter until it has been digested and excreted by insects (Birk 1979; Choudhury 1988), and high oil and polyphenol content of fresh leaves may deter insect feeding (Stone and Bacon 1994). Together these factors result in a resource of low nutritional value that breaks down slowly, and tends to build up on the floodplain when conditions are dry.

Fungal dynamics measured in the dry treatment were consistent with the current understanding of litter decomposition in arid environments. Where moisture is absent from the surface, litter decomposition proceeds mainly as a result of photo-degradation (Smith, Gao et al. 2009), and biological processes occur in pulses as a response to rainfall (Collins, Sinsabaugh et al. 2008). Under this paradigm, biological decomposition of surface litter ceases during the dry phase and fungal activity takes place beneath the soil surface. No loss of mass or organic matter was observed from E. camaldulensis under dry conditions. On the floodplain, such conditions would lead to litter accumulation (Glazebrook and Robertson 1999; Graça, Pozo et al. 2002). This accumulation of litter represents a reserve of carbon that can be utilised by fungi and other organisms with the next pulse of water (rain event or flood), in line with the pulse-reserve model of arid ecosystems (Noy-Meir 1973; Ogle and Reynolds 2004; Reynolds, Kemp et al. 2004).

Fungal activity at rates too slow to be measured is consistent with a fungal community that is dormant, and fungal DNA amplified by PCR may be derived either from live mycelium or viable propagules. This suggests the fungal communities colonising fresh and aged E. camaldulensis leaves are dormant under dry conditions, or growing at an
extremely slow rate. Such conditions occur seasonally in the floodplain wetlands of the Murray-Darling Basin under a non-regulated water regime, although they may be temporarily alleviated by rain events. The terrestrial and aquatic fungal communities may use dormancy as a mechanism for surviving these predictable periods of unfavourable conditions (Thomas 1996), inundation and drying, respectively. As wetland drying alleviates sediment anoxia, it is likely to stimulate the germination of terrestrial decomposer organisms within the sediments (Neckles and Neill 1994; Williams 2006). For aquatic species, drying may trigger reproduction, resulting in the production of dessication-resistant spores and conidia (Jennings and Lysek 1996). High temperatures during the dry period may also “activate” these dormant propagules, priming them for germination when conditions are suitable for growth (i.e. inundation) (Cooke and Whipp 1993). Sharp increases in fungal biomass on aged leaves early in the incubation period, followed by sharp declines are consistent with growth and sporulation, and may indicate a response to leaf drying.

Primary metabolism involves the acquisition of nutrients from the environment to use as energy sources or in order to produce substances such as proteins, lipids and nucleic acids (Isaac 1997). Fungal secondary metabolism is the production of new substances from these cellular compounds that are intended for use in interaction with the environment (Thrane, Anderson et al. 2007). Fungi may use secondary pathways, which may include producing agents of lignin degradation, in response to desiccation (Jennings and Lysek 1996). Thus, peaks in oxidative enzyme activity early in the incubation period may be a response to leaf drying, and not to the treatment conditions. As no significant mass loss was observed under dry conditions, it is likely that lignin and cellulose were not being mineralised to any great extent under this treatment. The observed activity reflects a response to moisture availability, as the enzyme assay measures processes in solution. As such, these data represent potential enzyme activity under moist conditions, and suggest that the fungal community on aged leaves is capable of responding quickly to moisture availability.

The drying of leaves has received critical attention (Boon 2006; Boulton and Boon 1991) as it is known to alter the chemical (Gessner and Schwoerbel 1989; Serrano 1992) and biological (Serrano and Boon 1991) properties of leaves. In this experiment, drying leaves at 50°C may have been sufficient to activate dormant fungal propagules which
later germinated during incubation. However, drying is essential to standardise leaf moisture content for gravimetric analyses. Drying at more moderate temperatures would therefore be the preferable approach where it is desirable to minimise impacts upon fungal community structure and biomass.

4.4.3 **Fungal activity under moist conditions - the terrestrial aging process**

Under terrestrial conditions with sufficient moisture, *E. camaldulensis* leaves on the floodplain are colonised by terrestrial fungi. The high levels of fungal biomass accumulated on both fresh and aged leaves in less than 30 days in the moist treatment suggests that the fungal communities on these leaves were undeterred by leaf defensive compounds. However, on fresh leaves the activity of enzymes that cleave sugars from cellulose, starch and hemicelluloses was close to zero, indicating inhibition of fungal activity towards these plant polymers. Inhibition is likely to be due to the presence of polyphenols, which would be rapidly leached from fresh leaves on the floodplain with rainfall (Serrano 1992). As rainfall was absent from the artificial environment of the mesocosm, the level of enzyme activity observed on fresh leaves may have remained low for a longer period than might be expected on the floodplain. While it would appear that aged leaves are a better fungal substrate than fresh leaves under moist conditions, these two leaf types represent two different stages in the terrestrial aging process that are likely to co-exist on the floodplain, with no adverse affects on the indigenous biota.

The process of “terrestrial aging” is known to produce chemical and physical changes in the decomposing leaf (Anesio, Tranvik *et al.* 1999; Baldwin 1999; Smith, Gao *et al.* 2009), such as a reduction in bio-available organic molecules and the loss of inhibitory substances (Canhoto and Graça 1996; Love 2005). Differences in fungal colonisation and activity on fresh and aged leaves indicate that the terrestrial aging process facilitates immediate colonisation of *E. camaldulensis* leaves by fungi when they enter the aquatic phase. Terrestrial aging leads to changes in fungal community structure, higher total fungal biomass, and enhanced enzyme activity towards cellulose in aquatic ecosystems, but losses of mass and organic matter are not significantly affected.

Under moist terrestrial conditions leaf mass loss is driven by leaching and biological processes (Coûteaux, Bottner *et al.* 1995). My results have shown that fresh leaves have low fungal biomass in the early stages of terrestrial decomposition, low rates of enzyme
activity towards cellulose, starch and hemicelluloses and negligible losses of mass and organic matter. This suggests that the fungal community present on fresh leaves, while tolerant of the leaf’s chemical defences, are not efficient in the breakdown of leaf structural carbohydrates and may be consuming other resources such as pectin, protein and free sugars. A change in community structure after 64-100 days brings with it increased activity towards cellulose, starch and hemicelluloses, and increased mass loss. Under laboratory conditions, aging of *E. camaldulensis* leaves to the point where fungi are not inhibited took 30 days. Under field conditions, where sunlight, leaching and litter organisms are more active, it is likely that this period would be shorter.

My results showed that oxidative enzyme activity towards lignin reached a peak during the first week of incubation for all treatments and for both leaf types. Lignin is less bio-available than other leaf components, and it is commonly accepted that litter decomposing micro-organisms utilise lignin after more bio-available leaf components are exhausted (Hammel, 1997). While lignin degradation is likely to be most prevalent in the soil, or at the base of the litter layer (Melillo, Aber *et al.* 1989), it is shown here to occur concurrently with hydrolytic activity towards cellulose, starch and hemicellulose. However, it is important to note that the magnitude of peaks in activity of lignin degrading enzymes observed early in this experiment may be partly due to responses of the fungal community to drying at high temperatures.

### 4.4.4 Aquatic decomposition processes

The levels of fungal biomass accumulating on submerged fresh and aged *E. camaldulensis* leaves were 3.7 and 6.2 mg.g⁻¹ litter dry mass, respectively, after 64 days. Pozo *et al.* (1998) reported that *E. globulus* leaves accumulated 65 mg fungal biomass.g⁻¹ litter dry mass in a Spanish stream, and similar values on the same leaves were reported from a stream in Portugal (Sampaio, Cortes *et al.* 2004). The biomass accumulating in the mesocosms was also low when compared to values reported from the leaves of other tree species submerged in rivers in the northern hemisphere (Gessner and Chauvet 1994; Gessner, Robinson *et al.* 1998; Hieber and Gessner 2002; Suberkropp 2001; Van Ryckegem, Gessner *et al.* 2007) and macrophytes in freshwater wetlands (Kuehn, Lemke *et al.* 2000; Kuehn and Suberkropp 1998a). Similarly low levels of biomass (5.8 mgC.g⁻¹ AFDM) have been recorded from macrophyte litter in a prairie wetland (USA) (Verma *et al.*, 2003), but fungal biomass on the leaves of *Acer rubrum* submerged in a woodland
wetland was ~5 times higher (28 mg.g\(^{-1}\) litter dry mass) (Inkley, Wissinger et al. 2008). It is possible that the artificial environment of the mesocosm has impeded the development of fungal biomass.

When fresh leaves were submerged, fungal biomass was initially low and remained low for one to two months, while aged leaves began to accumulate fungal biomass immediately, and moderate levels of biomass were maintained for a number of months. Lower fungal biomass implies lower food value (Canhoto and Graça 1995; Suberkropp, Arsuffi et al. 1983) and reduced palatability to invertebrates (Hladyz, Gessner et al. 2009). Thus a lag time in the colonisation of fresh leaves may have implications for wetland food chains, as the inundation of litter and sediments in floodplain wetlands is also likely to trigger emergence of aquatic invertebrates (Hillman and Quinn 2002; Jenkins and Boulton 2007). If floodplain aged eucalypt leaves are inundated when the wetland fills, insect larvae that feed on conditioned leaves will encounter abundant food resources. The inundation of fresh leaves will not provide a nutritious food resource for some months. Thus, if wetlands are inundated in summer when eucalypt leaves are freshly fallen, the conditioned leaves are likely to have lower fungal biomass, lower nitrogen content (Reid, Quinn et al. 2008), and be less nutritious for invertebrates. This will inhibit survival, growth and reproduction in emerging invertebrates (Albariño, Villanueva et al. 2008; Bärlocher 1985), influencing recruitment rates and reducing food resources for higher trophic levels. This phenomenon may play a role in changes in wetland invertebrate assemblages previously associated with river regulation (Hillman and Quinn 2002; Quinn, Hillman et al. 2000).

Inundation poses several challenges to fungal communities present on leaves. Oxygen availability is reduced, nutrients are removed from the substrate and competitive interactions between fungal species are altered. These challenges usually result in the decline of the dominant terrestrial species soon after inundation, and their replacement by species adapted to aquatic conditions (Boon 2006; Brinson, Lugo et al. 1981; Kuehn, Lemke et al. 2000). In terrestrial systems *Eucalyptus* leaves are typically colonised by fungal taxa such as Capnodiales and Pleosporales (Dothideomycetes), Eurotiales (Eurotiomycetes), Hypocreales and Chaetosphaeriales (Sordariomycetes), Mucorales and Mortierellales (Glomeromycetes) (Dix and Webster 1995; Eicker 1973; Macauley and Thrower 1966), while leaves in stagnant waters such as wetlands are commonly
colonised by Chytridiales (Chytridiomycetes) (Lefevre, Bardot et al. 2007),
Leptomitales, Saprolegniales and Peronosporales (Oomycetes) (Czeczuga, Mazalska et al. 2005; Czeczuga, Muszyńska et al. 2007), aquatic hyphomycetes (Dick 1976a; Pattee and Chergui 1995) and aero-aquatic hyphomycetes (mainly Ascomycetes) (Kjoller and Struwe 1980). Leptomitales, Saprolegniales and Peronosporales are Stramenopiles which colonise plant material soon after inundation, then decline in abundance as decomposition proceeds (Bärlocher 1992).

Amplification of fungal DNA by PCR indicates that fungal DNA is present but the method used is not a reliable measure of abundance, and does not distinguish between active mycelia and viable propagules. Species that colonise leafy substrates in terrestrial environments may die with inundation, or may remain alive but become inactive. Alternatively they may continue to decompose the leaf resource but discontinue their life cycle, or continue their life cycle but produce propagules that are not viable in the aquatic environment (Thomas 1996). All of these groups, along with fungi able to germinate, grow and reproduce in the aquatic environment, may have contributed to the total fungal DNA extracted from the decomposing leaf material. Thus, the finding that fungal communities present before inundation and after 100 days in the water are significantly dissimilar strongly suggests replacement of terrestrial species with aquatic species. Identification of the fungal species involved would be required to confirm such a transition. Alternatively, reverse transcription PCR would amplify only the DNA from active organisms.

Fungal community structure data, when considered with fungal biomass and enzyme activity data, suggest a 2-stage succession of fungal species on submerged leaves, similar to that reported by Verma et al. (2003). A terrestrial community is present prior to 16 days incubation, and is then replaced by an aquatic community. The biomass and enzyme activity of the terrestrial community (<16 days incubation) differed between fresh and aged leaves under aquatic conditions, but after 16-30 days the biomass and activity of the aquatic community and substrate organic matter losses were similar on both leaf types. Enzyme activity towards lignin was highest when the terrestrial community was dominant, then continued at moderate levels while the aquatic community was present.
The terrestrial fungal community exhibited higher enzyme activity towards cellobiose, starch and hemicellulose and lower activity towards cellulose under terrestrial conditions, as compared with aquatic conditions. However under aquatic conditions, it was the aquatic community that exhibited the highest activity rates for all of these polymers. This suggests that the aquatic fungal community is able to efficiently break down cellulose, starch, hemicellulose and lignin, perhaps more effectively than the terrestrial community on the same substrates.

The water regime has a strong impact upon litter decomposition and carbon dynamics in wetlands (Battle and Golladay 2001; Inkley, Wissinger et al. 2008; Neckles and Neill 1994; Ryder and Horwitz 1995). The results presented here show that fungal dynamics and mass loss due to leaching differ substantially when fresh and aged E. camaldulensis leaves are inundated, and these differences will influence water quality. Significant loss of mass and organic matter from fresh leaves during the first week in the aquatic phase is consistent with previous studies showing that much of the mass loss from inundated E. camaldulensis leaves is due to leaching (Briggs and Maher 1983; Day 1983; Glazebrook and Robertson 1999). Carbon leached from these leaves contributes to the level of DOC in the water column. Summer inundation of floodplain wetlands can produce DOC concentrations of around 15ppm, which is the twice that produced by spring inundation. These rapid increases in DOC have the potential to impact upon water quality in terms of colour, microbial activity, dissolved oxygen (Campbell and Fuchshuber, 1995) and nutrient concentrations (Baldwin 1999; O’Connell, Baldwin et al. 2000; Robertson, Bunn et al. 1999; Watkins, Quinn et al. 2010) and have other adverse impacts on wetland biota (Gehrke, Revell et al. 1993; Graça, Pozo et al. 2002; Watkins, Quinn et al. 2010). Anoxia may further inhibit fungal activity. These impacts could be expected to be smaller when aged leaves are inundated due to lower carbon losses via leaching, and lower bio-availability of the leachate (Baldwin 1999).

4.5 Conclusions

This experiment has shown that fungal community structure differs between fresh and terrestrially aged E. camaldulensis leaves, and that temporal changes in fungal biomass and enzyme activity differ in dry and moist terrestrial conditions, and aquatic conditions. Leaf mass loss differs between leaf types when moisture is not limiting, but the proportion of organic matter lost does not differ between fresh and aged leaves. These
results strongly suggest that a period of terrestrial decomposition is important for *E. camaldulensis* leaves as it promotes fungal colonisation, which is known to improve the food value of submerged leaves. Summer inundation of fresh leaves in floodplain wetlands will lead to reduced levels of fungal colonisation and leaf conditioning, potentially impacting upon food chains, water quality and carbon dynamics.

While the current experiment provides a model for the decomposition of eucalypt leaves in floodplain wetlands, the conditions in these habitats are more variable than those operating in a laboratory, with complex biological interactions. The artificial conditions in the mesocosms may also have had some influence on the amount of fungal biomass able to accumulate on the leaves. Thus, having demonstrated a strong response of the fungal community to moisture availability in leaf litter, in Chapter 5 I examine the response of fungal community structure and biomass to changing moisture availability in the sediments of a floodplain wetland as it undergoes seasonal drying.

Later chapters expand upon our understanding of fungal dynamics and leaf break down under field conditions. The accumulation of fungal biomass on floodplain aged *E. camaldulensis* leaves is compared between standing and flowing waters of lowland streams in Chapter 6. Chapter 6 also quantifies the contribution of stramenopilous organisms to the biomass accumulating on submerged leaves. Furthermore, as the enzyme activity data presented here have shown that lignin and polysaccharides are being degraded in submerged *E. camaldulensis* leaves, the chemical changes taking place within the submerged leaves as a result of this activity are examined in Chapter 7.
Fungi in Drying Wetland Sediments

5.1 Introduction

The results from the pilot study in Chapter 3 indicated that fungal DNA was present in the sediments of Normans Lagoon, and that the fungal community structure may change with sediment moisture content. Previously, analysis of microbial phospholipid fatty acids (PLFA) had indicated that fungal biomarkers were absent from the sediments of Ryans Lagoon, a similar wetland also on the floodplain of the Murray River (Boon, Virtue et al. 1996). In this chapter I explore fungal dynamics in the sediments of Quat Quatta East Lagoon, again on the Murray River floodplain.

The sediments of floodplain wetlands in the Murray-Darling basin are generally anoxic when the wetlands are holding water, and organic matter decomposition in the sediments is dominated by anaerobic bacteria (Boon 2000). In summer, the water column may become thermally stratified each day (Ford, Boon et al. 2002), leading to anoxia in the bottom layers, although the water column is generally mixed overnight. These anoxic conditions, combined with the knowledge that most filamentous fungi are aerobic and ergosterol was absent from the sediment s of a typical wetland, led Boon et al. (1996) to conclude that fungi were not important in organic matter processing in these systems. However, we have seen in the previous chapter that fungi are able to colonise submerged plant detritus in standing water, and fungi are also known as parasites and endophytes of algae and wetland macrophytes (Czeczuga, Mazalska et al. 2005; Kagami, de Bruin et al. 2007; Khan 1993; Lefevre, Bardot et al. 2007), which are abundant in these systems (Robertson, Bunn et al. 1999). As this plant and algal detritus often becomes buried in the sediments, it is logical to conclude that fungi might also be present in wetland sediments.

While wetland sediments are usually anoxic, the narrow sediment water interface zone may be oxic, and oxic zones may be created by plant roots or burrowing animals. For example, Qiu et al. (2005) found that while the proportion of respiration in wetland sediments due to fungi was significantly lower than in leaf litter, fungal activity was present and was higher than that found in upland soils. However, this aerobic activity is likely to be confined to the sediment surface, where the greatest proportion of aerobic organic matter decomposition occurs (Adams and van Eck 1988).
Fungal DNA has also been isolated from anoxic sediments in sulfide-rich springs and a freshwater pond (Luo, Krumholz \textit{et al.} 2005). These fungi were mainly Basidiomycete and Ascomycete yeasts, but chytrids were also present. Luo \textit{et al.} (2005) also detected ergosterol and fungal PLFAs in these sediments. Seo and DeLaune (2010) recorded fungal respiration under highly reducing conditions in sediments from a forested wetland. Hallett and Dick (1981) found that the diversity of Oomycete propagules in the littoral mud of a small freshwater ornamental lake in the United Kingdom was the same as that in the water column, although propagule abundance was lower. Anoxia is also known to inhibit the lignin degrading enzymes of fungi (Freeman, Ostle \textit{et al.} 2001) but lignin degradation has been shown to occur in the anaerobic sediments of a freshwater marsh (Okefenokee Swamp, southern Georgia, USA) (Newell, Moran \textit{et al.} 1995).

As sediments dry out the anoxia is relieved, water is less available and the chemical environment of the sediments changes (Baldwin and Mitchell 2000). Such extreme changes are likely to alter the decomposer community. Changes in fungal community structure (Toberman, Freeman \textit{et al.} 2008), increased biomass (Jaatinen, Fritze \textit{et al.} 2007) and increased respiration (Qiu, McComb \textit{et al.} 2005) have been reported. Rinklebe and Langer (2006) found that fungal PLFA were absent from floodplain soils that had been inundated for long periods, but not from soils from higher positions on the floodplain. However, exposed sediments have different properties to soils, and maximum microbial biomass may be concentrated in the layer 10-30cm beneath the surface, instead of in the surface layers as is the case for soils (Qiu, McComb \textit{et al.} 2003).

There are no published reports of a concurrent investigation of fungal community structure and fungal biomass in an Australian floodplain wetland, although biomass has been inferred from sediment induced respiration measured in a coastal freshwater wetland under conditions that inhibit prokaryotes (Qiu, McComb \textit{et al.} 2005). While the pilot study detailed here (Chapter 3) has shown that fungal DNA is present in wetland sediments, the taxa comprising the fungal community are unknown. It is also unknown whether the fungal DNA is derived from active mycelium or dormant propagules.

As many of the floodplain wetlands in the Murray-Darling Basin are ephemeral, the response of the sediment fungal community to drying and re-wetting will impact upon
the rate of organic matter respiration and food web interactions. We have seen differences in the fungal community structure between wet and dry sediments (Chapter 3), but the sediment moisture content required to produce a response from the fungal community, and the time taken to respond to these changes are unknown.

In this chapter I report on a field experiment where fungal community structure and biomass were measured in a floodplain wetland as it underwent summer drawdown. The sediments were sampled over 35 days during drawdown, and further samples were taken after 2 months of inundation (90 days from day 1). The moisture content, organic matter content and fungal biomass was determined for samples taken over a 2.5m wetness gradient in the exposed wetland sediments. Spatial and temporal changes in fungal community structure within the transect were assessed using T-RFLP, and these changes were related to the moisture and carbon content of the sediments. I hypothesised that a change in the fungal community composition consistent with a change from an aquatic community to a terrestrial community would be observed between wet and dry points on the gradient, and at the same sediment position over time. Such a change would be accompanied by increased fungal biomass.

5.2 Materials and Methods

5.2.1 Site description

Quat Quatta East Lagoon connects with the north bank of the Murray River approximately 5km west of Howlong, NSW (35° 58′ 19.82″ S, 146° 34′ 44.67″ E, Elevation 150m elevation) (Figure 5.1). The Murray River is highly regulated (Maheshwari, Walker et al. 1995), supplying water for irrigation, stock usage and domestic supply over the warmer months, while flows in cooler months are retained in storages. The water level in Quat Quatta East lagoon decreased between 5 December 2008 and 26 December 2008 until the wetland had contracted to three small pools (Figure 5.3, (a)). The drying event led to the death of a large amount of fish, and a blue-green algal bloom (Figure 5.2, (d)). Inflows commenced in late December 2008, and all bare sediments were inundated by March 2009 (Figure 5.3, (b)). The riparian vegetation at Quat Quatta East Lagoon is dominated by E. camaldulensis open forest with a grassy understory, with reeds, sedges and rushes along the margins of the lagoon. The area is grazed by cattle (Figure 5.2, (c)) and used for camping and recreational fishing.
5.2.2 Sample collection

One 2.5m transect was established in the sediments of Quat Quatta East lagoon as it underwent drying between December 2008 and February 2009. The transect was oriented perpendicular to the water line, and positions 50 cm apart were marked out using wooden pegs. The position furthest from the centre of the water body was denoted as position 1, and positions were numbered consecutively up to position 6 (as pictured in Figure 5.2, (a)). The sediments were sampled on five occasions. The moisture status of the sediments, i.e. whether they were exposed, submerged or transitional (edge), at the 5 sampling dates is shown in Table 5.1. On 12 December 2008 exposed sediments were damp on the surface due to light rain. After 35 days increased flows in the Murray River for the purposes of downstream irrigation led to inflows to the wetland, and all 6 positions were submerged (Figure 5.2, (b)).

On each sampling occasion, the upper 5 cm of sediment at each position was collected into a sterile 70 ml specimen jar. On 7 March 2009, additional reference samples were
taken from the dry and vegetated soil above the high water line of the wetland (bank), the reed bed at the margin of the wetland, and positions 1 and 2. On this occasion, all of the original six positions were submerged beneath more than 1m of water.
Table 5.1: The moisture status of sediments at 6 positions over a 35 day period.

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Figure 5.2: Positions were marked along a transect in the exposed sediments. Position 1 was exposed at the commencement of the 35 day period, while position 6 was inundated. Panel (a) shows the water level after 8 days while panel (b) shows the water levels after inflows began on Day 35. The wetland was frequently visited by grazing cattle (c) and dead fish and algal blooms were observed as the wetland approached dryness (d).
Figure 5.3: The water level in Quat Quatta East Lagoon as it approached dry conditions (a) and after it had received inflows from the Murray River (b). In the background, the dominant tree species, *E. camaldulensis*, can be observed, while reed beds and immature *E. camaldulensis* are seen in the foreground.

5.2.3 Sediment moisture and organic matter content

Three analytical replicates of sediment samples were homogenised by mixing, then 10-15 ml of sediment was weighed into pre-fired porcelain crucibles (wet mass - WM) before being dried at 105°C for 72 hours. Crucibles were cooled in a desiccator, before being re-weighed (dry mass - DM). Following this, sediments were fired in a muffle furnace for 1 hour at 550°C, cooled, and re-weighed (ash-free dry mass - AFDM). Moisture content was calculated as the difference between WM and DM and expressed as a percentage of WM. Organic matter content was calculated as the difference between DM and AFDM and expressed as a percentage of DM. Moisture content and organic matter content data was analysed using 2-way ANOVA (without replication) on the mean values for each position in each time. Correlation between these variables was assessed using multiple linear regressions. Data analyses were carried out using Microsoft Office Excel (2003, SP3, Microsoft Corporation, Redmond, Washington, USA.).

5.2.4 Microscopy

Wetland sediment from each sample position was smeared onto a glass microscope slide and stained with lacto-phenol cotton blue for 5 minutes prior to examination. These slides were examined at 40×, 100× and 400× magnification using an Olympus BX 51 compound microscope (Olympus Australia, Pty. Ltd., Oakleigh, Victoria, Australia) equipped with an Olympus DP12 digital camera (Olympus Australia, Pty. Ltd., Oakleigh,
Victoria, Australia). Images were processed using Olysia software (Soft Imaging System, Münster, Germany).

In the absence of cultured specimens, reproductive features, and knowledge of the cell wall composition or microstructure it is not possible to make definitive classifications of the fungal tissues observed. However, it is possible to make a preliminary identification based on the features of higher level taxa. Higher level identification of fungal hyphae was made according to the key published in Fungi of Australia (Walker 1996), and with reference to Thomas (1996). The more recent key published by Adl et al. (2005) places the fungi within the Super-group Opisthokonta, and subdivides fungi into Ascomycetes, Basidiomycetes, Chytridiomycetes, Glomeromycetes, Microsporidia, Urediniomycetes, Ustilaginomycetes and Zygomycetes. The Stramenopiles are placed within the Super-group Chromalveolata, and the group formerly referred to as Oomycetes are newly classified as Peronosporomycetes. However, this key relies on the identification of cell organelles, the metabolic pathways used by the organisms and the composition of the cell walls. Therefore, the earlier classification system has been used in these preliminary descriptions of the organisms observed.

Hyphae have been identified as having (a) regular septa with adjacent clamp connection (Basidiomycetes, Urediniomycetes, Ustilaginomycetes), (b) septa but no clamp connections (Ascomycetes, and some Chytridiomycetes), or (c) no septa (coenocytic) (Zygomycetes, Chytridiomycetes, Oomycetes). Microsporidia and Glomeromycetes are obligate internal parasites and are not expected to be found in wetland sediments.

Additional references were used for preliminary identification of zoospores and encysted zoospores (Dick 1969; Dick 1972; Hallett and Dick 1986; Pickering, Willoughby et al. 1979).

5.2.5 T-RFLP

The procedure used to extract DNA from wetland sediments, amplify the DNA using PCR, digest to create terminal restriction fragments, and analyse fragment length data has been detailed in section 3.2.3.
5.2.6 **Fungal biomass - ergosterol content**

Ergosterol content in wetland sediments was assessed using saponification followed by solid phase extraction and the reversed-phase liquid chromatography isocratic method with UV detection, similar to that described in Chapter 4 (Gessner 2005). However, minor modifications were required to accommodate the change in substrate from leaf discs to sediment.

Briefly, ~1g of fresh sediment was preserved in 8 ml of methanolic potassium hydroxide (KOH/MeOH, 8g/L) and stored at 4°C in the dark. On the day of extraction, sediment was re-suspended in the storage solution and decanted into 50ml Pyrex tubes. The storage tube was rinsed with a further 2 x 1ml of KOH/MeOH, which was added to the 50 ml tube. A control was comprised of 1g of sediment that had been fired in the muffle furnace at 550°C for 1 hour, and then allowed to cool. To this, 250 µL of ergosterol standard in KOH/MeOH was added. The sediments were saponified under reflux for 30 min at 80°C, then allowed to cool for a further 20 minutes.

Sediments were then resuspended before being transferred into a disposable 10ml polypropylene centrifuge tube and centrifuged at 3000 rpm for 5 minutes in an Allegra X-15R centrifuge (Beckman-Coulter, Fullerton, California, USA). The supernatant was then transferred into the Sep-Pac cartridge using a pipette. A further 1 ml of methanol was added to the centrifuge tube, the sediment was resuspended by vortexing, and then centrifuged at 1000 rpm for 30 seconds. The supernatant was transferred to the Sep-Pac cartridge, and then the washing step was repeated. A total of 12ml of solution was transferred.

Additional analysis steps were the same as for leaf disc ergosterol as detailed in Section 4.3.2.

Ergosterol detection limits are a function of the minimum peak area of 4930 used in the integration method. This corresponds to 0.2 µg ergosterol and approximately 0.85 µg ergosterol .g⁻¹ sediment DM. This equates to approximately 5mg fungal biomass.g⁻¹ sediment DM.
5.3 Results

5.3.1 Sediment moisture content

The moisture content of wetland sediments sampled over 35 days of drawdown differed significantly between positions and times (ANOVA: P<0.001 for both variables) (Figure 5.4). Saturated wetland sediments contained 50-60% moisture, and this figure fell to 15-45% moisture with exposure and subsequent drying. None of the sediments could be said to be desiccated prior to inundation after 35 days. For samples from day 90, the mean moisture content was 1.11% for the sediment (soil) sampled from the bank, 32.25% from the reed bed, 30.63% from position 1, and 28.03% from position 2.

![Figure 5.4: The mean moisture content of wetland sediments over a period of wetland drying and subsequent re-wetting. Position 1 (indicated by a white upright triangle) is furthest from the centre of the water body, while position 6 (indicated by a black inverted triangle) is closest to the centre of the water body. Positions 5 and 6 were submerged until exposure on day 22. All samples were exposed on Day 22, while all samples were inundated on Day 35. Error bars indicate the standard error of each data point, with maximum standard error being 2.8%.](image)

5.3.2 Sediment organic matter content

The organic matter content of wetland sediments over the drawdown period is shown in Figure 5.5. Organic matter content differed significantly between positions (ANOVA: P=0.01) but not between times (ANOVA: P=0.40). While there was a trend for deeper sediments to have higher organic matter contents, this was not consistently the case.
Similarly, there was a trend towards decreasing organic matter upon sediment exposure, and increases with inundation, but this was not seen consistently. Increases in the sediment moisture content were correlated with increased sediment organic matter but the relationship was not strong (Multiple Linear Regression: $R^2 = 0.47; P<0.001$).

![Figure 5.5: The organic matter content of wetland sediments. There is a trend towards decreasing organic matter with drying, and increasing organic matter with re-wetting, but this pattern is not consistent across all sediment positions. Error bars indicate standard error about the mean of each data point.](image)

5.3.3 **Fungal biomass – ergosterol content**

None of the sediment samples examined contained detectable quantities of ergosterol.

5.3.4 **Microscopy**

As no ergosterol was extracted from the wetland sediments, the sediment samples were examined under the microscope for evidence of fungal organisms. Evidence of fungal hyphae (Figure 5.6) and propagules (Figure 5.7) was found but their distribution throughout the sediments was sparse. Closer to the centre of the wetland, the hyphae observed were mainly aseptate, and fungal hyphae were often observed within diatoms (Figure 5.7, (d)). While aseptate hyphae may belong to the Zygomycetes, Chytridiomycetes or Oomycetes, the septate hypha with a clamp connection (Figure 5.6 (d) and Figure 5.7 (d)) indicates a Basidiomycete.
Encysted propagules were similar to those in the Oomycete families Saprolegniaceae and Pythiceae, although magnification and image resolution was not sufficient to discern the presence or absence of hairs or spikes in order to classify these propagules to genus or species.

Figure 5.6: Hyphae observed in sediment samples. Shown are aseptate hyphae that may be Chytridiomycetes, Zygomycetes or Oomycetes (a, b and c), and a septate hypha with a clamp connection (CC) indicating a species belonging to the Basidiomycetes (d). 400x magnification. Scale bar = 50µm.
Figure 5.7: Fungal propagules found in sediment samples (a, b and c) and a fungal hypha enclosed within a diatom frustule (d). The propagule shown in panel (a) is an encysted Oomycete propagule and a similar specimen is seen germinating in panel (b). The organism adjacent to the propagules (arrow) appears to be an amoeba. The presence of a prominent oil droplet (arrow) suggests the propagule in panel (c) is a chytrid sporangium. 400x magnification. Scale bar = 50µm.

5.3.5 T-RFLP

Changes in the fungal community structure over time were not significant between positions (Two-way ANOSIM R=0.208; P=0.001) or times (Two-way ANOSIM R=0.347; P=0.001). Figure 5.8 shows that the fungal community structure changed randomly between days 1 and 35 at position 1. This area remained dry until it was inundated at day 35. After 35 days of inundation there was a change in community structure indicated by movement to the right of the ordination. This change corresponded
to a decrease in the mean number of terminal restriction fragments (T-RFs) obtained, from 24.3 at day 1 to 10.7 at day 90.

The fungal community structure changed with the moisture content of the sediments. Figure 5.9 shows that dry samples (0-10%) were dissimilar to saturated (50-60%) (ANOSIM Pairwise test: R=0.803; P=0.001), and very wet (40-50%) samples (One-way ANOSIM Pairwise test: R=0.806; P=0.002) with no significant difference observed between the intermediate moisture categories (One-way ANOSIM Pairwise test: R<0.584). The fungal community structure did not differ significantly between inundated and exposed sediments (One-way ANOSIM: R=0.101; P=0.019). Higher mean numbers of T-RFs were seen in samples with 15-45% moisture content (Dry samples have 8.83 T-RF, saturated samples had 14.45 T-RF and sediments with 15-45% moisture had 17.39 T-RF). No relationship was found between fungal community structure and sediment organic matter content (One-way ANOSIM: R=0.184; P=0.001).

Samples taken at day 90 (Figure 5.10) show that the fungal community structure differed between the bank (never inundated), the reed bed (recently inundated), and the sediments at positions 1 and 2 (inundated for 55 days). The largest differences were found between position 2 and the reed bed (One-way ANOSIM: R=0.692; P=0.018) and between position 1 and the bank (One-way ANOSIM: R=0.611; P=0.012). These differences were associated with moisture content and the number of fungal amplicons. The reed bed samples yielded a larger number of T-RFs (Mean 14, max 19), compared to the deeper sediments (Mean 9.2, max 15) and the bank (mean 8.8, max. 13).

When the T-RFLP data from positions 1, 3, 6 and the dry bank were analysed using group average cluster analysis the exposed and inundated samples were dissimilar (Figure 5.11). Position 1 was exposed on all days except day 35. These samples were generally clustered together, with the exception of a day 22 replicate (when these sediments were driest) that clustered with bank sediments, a day 35 replicate (re-wet) that clustered with position 3 sediments and a day 22 replicate that clustered with position 6 sediments. Position 6 was wet on all days except day 22. Only one day 22 replicate separates from other position 6 samples. Inundated samples from position 3 (Day 1 and 8) clusters with position 6, while later samples (drying, dry and re-wet) cluster with
position 1. These data suggest that changes in fungal community structure may lag behind sediment inundation or exposure by more than 1 week.

Figure 5.8: The trajectory of change in fungal community structure at position 1 over time. Error bars indicate standard error about the mean of three points for each time. Axes are relative positions in data space and have no units.

Figure 5.9: MDS plot of sediment fungal community structure shows that there is some separation of groups by sediment moisture content. Symbols indicate different moisture content classes (see legend). Overlaid circles are intended to illustrate grouping of samples by moisture class, but have no statistical meaning.
Figure 5.10: MDS plot of T-RFLP samples from Quat Quatta sediments on Day 90. Dry sediments are shown by open symbols; wet sediments are shown by solid symbols. Sediments that had not been wet (Bank samples – see legend) separate well from sediments in the reed bed that had recently been submerged (circles), and those from positions 1 and 2, that had been submerged for 55 days (upright and inverted triangles, respectively). The circles are added to illustrate grouping only, and do not indicate statistical similarity.
Figure 5.11: Cluster analysis of the fungal community structure data from positions 1, 3 and 6 (with always dry controls from day 90 added as a reference) indicates strong grouping according to moisture availability, assuming some lag time. Position 1 was dry on all days except day 35. Position 6 was wet on all days except Day 22. Position 3 clusters with position 6 on days 1, 8 (when it was submerged) and 15 (when it was drying), and clusters with position 1 on days 15, 22 (drying) and 35 (re-submerged). One of the position 1 samples from day 22 is positioned with the always dry sediments. At this point it had been dry for 3-4 weeks.
5.4 Discussion

5.4.1 Fungal organisms are present in wetland sediments

While fungal biomass in the sediments of Quat Quatta East Lagoon was below detection limits (5mg biomass per gram sediment dry mass), fungal DNA could be amplified and microscopic examination of the sediments showed that both hyphae and propagules were present, but in low abundance. The organisms present consisted mainly of coenocytic hyphae, encysted propagules, and fungal tissues encased within diatom frustules.

Together, coenocytic hyphae and encysted propagules suggest that Oomycetes are present in the wetland sediments. These organisms and their propagules are able to survive hypoxic conditions in the surface sediments (Field and Webster 1983; Johnson, Seymour et al. 2002), and are able to utilise a range of substrates ranging from living and dead fish to plant detritus (Al-Rekabi, Al-Jubori et al. 2005; Czeczuga, Kozlowska et al. 2002; Czeczuga, Muszyńska et al. 2007; Pickering, Willoughby et al. 1979). Oomycetes do not have ergosterol in their cell walls, and thus would not be detected using the ergosterol method to measure fungal biomass. Nechwatal et al. (2008) were able to identify 18 Oomycete species, and found that permanently flooded sediments had a more diverse Oomycete community than drier sediments and that only 4 species were found in both flooded and dry sediments.

The hypha observed within the diatom frustule was septate with a clamp connection, indicating that it belonged to the Basidiomycetes. It is not clear whether this organism was an algal parasite or a decomposer of dead algal cells. Reports of fungi parasitising diatoms in freshwater systems commonly refer to Chytridiomycetes species (Bruning 1991; Kagami, de Bruin et al. 2007). While there are no reports of Basidiomycetes parasitising algae in freshwater systems, Basidiomycetes have been found to parasitise red algae in marine systems (Porter and Farnham 1986). It is therefore likely that the organism observed is saprobic, as Basidiomycete saprobes are known among the aquatic hyphomycetes (Ingold 1961; Nawawi 1985), in soils (Gams 2007) and marine sediments (Burgaud, Arzur et al. 2010).

Chytrid parasites were not observed on diatoms in the sediments. These fungi are common in lentic systems (Czeczuga, Kozlowska et al. 2002; Ellen Van Donk 1983;
Lefevre, Bardot et al. (2007) and are also important in the decomposition of organic matter, and conversion of inorganic carbon into organic carbon (Gleason, Kagami et al. 2008). However, their distribution is limited to fresh water with NaCl concentrations of <2%, and their growth is known to be inhibited by low dissolved oxygen concentration and temperatures above 30°C (Gleason, Kagami et al. 2008). These factors indicate that Chytrids are unlikely to be active parasites of diatoms in drying floodplain wetlands over the summer. Even when salinity is very low, temperatures regularly exceed 30°C and hypoxic conditions may occur (<2 mg O₂/L) (Smith 2010). Thus this ecological niche is unoccupied, and the biomass of dead diatoms is available to other fungi, such as soil basidiomycetes.

If Ascomycete or Basidiomycete yeasts were present in the sediments in any significant way, then their biomass would have registered as ergosterol, as these organisms tend to have higher concentrations of ergosterol in their cell walls (Pasanen, Yli-Pietila et al. 1999). Hyphae indicative of the Ascomycetes were also absent from the wetland sediments. The aquatic and aero-aquatic hyphomycetes are comprised mainly of species in this division, and their absence suggests that sediments are not a suitable substrate for these groups.

5.4.2 Changes with sediment exposure and inundation

The structure of this fungal community was seen to change with the moisture content of the sediments. While sediments remained either exposed or inundated, no change was observed in fungal community structure over space or time. However, the diversity of fungal amplicons decreased with both drying and saturation. Maximum amplicons diversity at 15-45% moisture content suggests that the fungal community requires a balance between moisture and oxygen availability. At any specified location within the wetland, such conditions will be limited to brief periods during drying and re-wetting of the sediments, and the response of the fungal community to sediment exposure may be delayed by more than a week.

While fungal community structure in soils is driven mainly by seasonal factors (Toberman, Freeman et al. 2008), community structure in wetland sediments appears to change with inundation and drying (Rinklebe and Langer 2006). Differences in fungal community structure between inundated wetland sediments and floodplain soil from the
bank suggests that an aquatic fungal community exists, and that it differs substantially from the terrestrial fungal community, with maximum amplicon diversity occurring during the transition between these two community structures.

While changes were observed in fungal community structure, no increases in fungal biomass were found. There are several possible reasons why this might be the case. Firstly, wetland sediments represent a harsh environment for fungi where oxygen may be unavailable, inhibiting their ability to degrade carbon sources with oxidative enzymes (Jennings and Lysek 1996; Wetzel 1992). Under these conditions fungi may not compete successfully with bacteria and fail to achieve growth (Menge and Sutherland 1987). Secondly, it may take longer than 35 days for the sediment fungal community to germinate and grow. Regular non-catastrophic disturbances such as drying events are known to stimulate greater diversity (Connell 1978; Grime 1973; Lake 2000). However, it may take some time for the biota to recover from such a disturbance (Boulton and Lake 1992). Thirdly, the propagules in the sediments may require a stimulus to germinate that they did not receive, such as a nutrient or hormone. Lastly, the fungal organisms present in the sediments may not contain ergosterol in their cell walls (e.g. Oomycetes and Chytrids), and therefore their biomass would go undetected.

5.4.3 Substrates for sediment fungi

The organic matter content of the sediments of Quat Quatta East Lagoon was 2-7%, which is low when compared with 10-26% reported in a similar wetland (Boon, Virtue et al. 1996), and only a proportion of this will be biologically labile (Baldwin 1999). However, in a drying wetland, there are a range of possible substrates that may accumulate on the sediment surface. These will include phytoplankton and animal biomass. When warm weather leads to evaporative concentration of nutrients and wetland drying both of these carbon sources will be deposited on the sediment surface. Dying fish can contribute a significant amount of carbon towards the productivity of waterholes in arid environments (Burford, Cook et al. 2008). These resources will be colonised by fungi if there is sufficient oxygen and moisture to sustain their growth.

5.4.4 Method considerations

While ergosterol concentrations in the sediments of Quat Quatta East lagoon were below 0.2 µg ergosterol.g⁻¹, it has been extracted from aquatic sediments in other studies. For example, ergosterol concentration was measured at 60-70 µg.g⁻¹ and 240 µg.g⁻¹ in
sulfide–rich spring and freshwater pond sediments, respectively (Luo, Krumholz et al. 2005), and 39 µg ergosterol g⁻¹ AFDM was found in the sediments of Canadian prairie wetlands (Headley, Peru et al. 2002). These results do not represent high levels of fungal biomass, but it may be that the fungal organisms in the sediments of many wetlands are not detectable using this method.

Oomycetes and Chytridiomycetes do not contain ergosterol, and are therefore their biomass cannot be quantified using this method (Gessner, Bauchrowitz et al. 1991; Newell 1992). If Oomycetes, Chytrids and their propagules comprise a significant component of the sediment fungal community, then ergosterol analysis will underestimate the fungal biomass present. However, these organisms will contribute DNA to fungal community structure analysis. Similarly, silicate diatom frustules may prevent ergosterol extraction from hyphae within them, while the procedure used to extract DNA is likely to rupture these structures. Ergosterol concentrations can also be expected to be lower at reduced oxygen tensions as the synthesis of ergosterol requires molecular oxygen (Safe 1973).

Ravelet et al., (2001) demonstrated that exposure of sediments to UV light, such as occurs with wetland drying, results in the photodegradation of ergosterol to ergocalciferol (vitamin D2). This is also true for ergosterol contained in dead fungal tissues (Mille-Lindblom, Wachenfeldt et al. 2004), but melanins in the fungal cell wall protect ergosterol in living fungi from UV light (Butler and Day 1998). Therefore, the ergosterol content of exposed sediments is a valid representation of living fungal biomass, and if samples are stored correctly (Newell 1995) exposure to UV light is not likely to influence sediment fungal biomass assessments.

The moisture content and organic matter content of the sediments are correlated to some extent because saturation reduces oxygen availability, and organic matter is decomposed at different rates in oxic and anoxic sediments. If the depth of the oxic sediment layer varied spatially, for example with differences in sediment texture, mixing the sediments would cause variation in the relative percentages of moisture and organic matter. In future work, these variables may be measured more accurately by taking a sediment core and assessing moisture and organic matter content, as well as fungal biomass and community structure, separately for the oxic and anoxic layers.
5.4.5 Ecological implications

As the fungal community in the wetland sediment was not found to change over 35 days while inundated, or alternatively while exposed, but changed with distance from the shore, then it is likely that the community was dormant (or growing very slowly) and composed mainly of propagules. Given low ergosterol results, it is likely that the propagules were derived from Oomycetes and Chytridiomycetes. Changes in community composition after 90 days are likely to be due to the deposition of fungal organisms and propagules from the inflowing water.

Many species belonging to the Chytridiomycetes are parasitic on phytoplankton, and these infections may play a pivotal role in the collapse of algal blooms (Ellen Van Donk 1983). Fungal infection of diatom hosts is favoured under conditions of high light intensity, high temperatures, and high host density (Bertrand, Coute et al. 2004). Some diatom species survive dry periods and periods of low abundance by forming “seed banks” in the sediments. However, infected algal cells have been found in the sediments when there is no sign of infected cells in the water column (Kagami, de Bruin et al. 2007), so diatoms in the sediments may act as a seed bank for fungal organisms as well.

Fungal parasites of algal often feed upon algae that are too large for zooplankton to consume (Kagami, de Bruin et al. 2007), but when their zoospores are released into the water column they provide a food source for Daphnia (Kagami, Van Donk et al. 2004). This link in the trophic relationships between wetland organisms helps to ensure that primary production is not lost when the larger algal cells fall to the sediment surface on senescence (Kiørboe 1993). This suggests that parasitic fungi may have an important function in pelagic food webs in wetlands (Lefevre, Bardot et al. 2007) and has been described as the “Mycoloop” by Kagami et al. (2007).

5.5 Conclusions

The fungal community in wetland sediments may have greatly reduced activity when (i) sediments have been inundated for an extended period, due to low oxygen availability, or (ii) when sediments are exposed and dry, due to low moisture availability. Oxygen and moisture availability may be more suitable for fungal organisms during transitional periods such as flooding and drying, in shallow water or where vegetation is established. However, the harsh chemical conditions in a drying wetland and the short duration of
these conditions at any point on the sediment surface severely limit opportunities for fungal growth. It is not yet clear what proportion of the changes observed in community structure are a response to higher temperatures, increased concentrations of dissolved nutrients and other substances, oxygen concentration or moisture availability. In floodplain wetlands, the fungal community in the sediments is likely to comprise a “seed bank” of propagules that are available to repopulate the water column after drying and refilling when suitable substrates, such as phytoplankton and fish, are available and environmental conditions are optimum. In future work, the importance of fungal parasites and saprobes in controlling algal blooms and utilising algal biomass should be considered in Australian lentic systems.

It would be beneficial to confirm the identity of the fungal taxa present in the sediments of floodplain wetlands, so as to better understand their ecological functions. Such investigations might involve growing cultures of the species present and sequencing their DNA. However, the low fungal biomass in the wetland sediments indicates that the impact of sediment fungi upon wetland carbon cycles is likely to be minimal, even though they may be important in sustaining pelagic food webs.
Fungal biomass accumulation on *E. camaldulensis* leaves differs between rivers and wetlands

6.1 Introduction

In the mesocosm experiment (Chapter 4) the amount of fungal biomass accumulating on submerged *Eucalyptus camaldulensis* leaves was 10-fold less than that reported on *E. globulus* leaves in Spanish and Portuguese stream ecosystems. Such a large difference in fungal biomass is likely to have significant impacts on aquatic food chains and carbon cycles. In this chapter, the accumulation of fungal biomass on *E. camaldulensis* leaves is compared between river and wetland sites, and possible relationships between fungal biomass, fungal diversity and the food value of leaf detritus are explored.

Fungal biomass can act as an important food source for aquatic invertebrates such as snails (McMahon, Hunter *et al.* 1974; Newell and Bärlocher 1993) and insect larvae (Bärlocher 1981; Bärlocher 1982; Bärlocher 1985), and form the base of aquatic food chains (Reid, Quinn *et al.* 2008). Fungal biomass is either removed from the leaf surface, or the leaf itself is consumed. Ecological interactions such as this may limit opportunities for fungal growth and reproduction, and influence standing biomass on submerged leaves. Chung and Suberkropp (2009) demonstrated that caddisfly larvae were only able to grow on leaves that were colonised by the aquatic hyphomycete *Anguillospora filiformis*, and early instar caddisfly larvae relied almost entirely on consuming fungal biomass to fuel daily growth.

The variables most likely to influence the magnitude of fungal biomass accumulation in aquatic systems include the quality of the litter substrate, the structure of the fungal community, environmental conditions and interactions with other organisms. The interaction of these variables over space and through time will result in variation in the pattern of biomass accumulation between locations, seasons, and with changes in flow and water quality.

The influence of substrate quality on fungal biomass accumulation was discussed in Chapter 4 and reviewed by Gulis (2001). Substrate quality for submerged plant detritus has been defined as the chemical composition of the decomposing material (Melillo, Naiman *et al.* 1984) and is often described in terms of nutrient concentrations (carbon,
nitrogen and phosphorus). Alternatively, the percentage composition of the major classes of plant bio-polymers (i.e. proteins, lipids, polysaccharides and lignin) may also infer substrate quality (Chamier 1985). These factors are also known to have a strong influence on the rate at which a substrate is decomposed (Melillo, Aber et al. 1982; Taylor, Parkinson et al. 1989). Fungal colonisation is known to enrich leaves with protein and nitrogen (Bärlocher 1985), and low ratios of carbon to nitrogen are correlated with nutritious food sources (Boyd and Goodyear 1971; Hladyz, Gessner et al. 2009; Taylor, Parkinson et al. 1989). Eucalyptus leaves colonised by fungi have been shown to be a potential source of nutrition for consumers (Canhoto and Graça 1996; Reid, Quinn et al. 2008; Schulze and Walker 1997), therefore changes in fungal biomass on submerged leaves is likely to impact upon higher trophic levels in aquatic environments.

Patterns of biomass accumulation observed in Chapter 4 suggest that biomass fluctuates with succession between early and late colonisers of the submerged leaf. This pattern consists of rapid growth following inundation, a reduction in biomass that may be related to sporulation events, and a later phase of growth and biomass loss. These two growth phases correlate temporally with early and late colonisation by different fungal communities, which may both be present on the substrate during a period of transition. Other studies have shown changes in biomass with change in community structure (Medeiros, Pascoal et al. 2009b; Van Ryckegem, Gessner et al. 2007).

Fungal biomass and community composition on submerged detritus can change with location due to changes in the riparian vegetation (Bärlocher and Graça 2002; Findlay, Howe et al. 1990; Graça, Pozo et al. 2002; Shearer and Webster 1985) and may differ between flowing and non-flowing aquatic environments (Baldy, Chauvet et al. 2002; Pattee and Chergui 1995). Environmental conditions such as temperature (Bärlocher, Seena et al. 2008), salinity (Hyde and Lee 1995; Roache, Bailey et al. 2006) and pH (Bärlocher and Rosset 1981; Iqbal 1976) are known to influence fungal community structure (Fischer, Bergfur et al. 2009; Nikolcheva and Bärlocher 2005) while dissolved nutrients and oxygen influence both community structure (Field and Webster 1983; Medeiros, Pascoal et al. 2009b) and biomass production (Gulis, Ferreira et al. 2006; Gulis and Suberkropp 2004; Sridhar and Bärlocher 2000; Suberkropp 1998). High concentrations of ambient nitrogen may provide an opportunity for fungi colonising
substrates low in nitrogen to accumulate biomass and thereby enrich the substrate with nitrogen (Stelzer, Heffernan et al. 2003).

The fungal community within decomposing leaf material in the aquatic environment co-exists with other organisms, including bacteria and Oomycetes. Fungi may compete with these organisms to obtain nutrients from the leaf biomass. Therefore, the abundance of such organisms might influence the ability of aquatic fungi to grow and accumulate biomass (Gulis and Suberkropp 2003; Mille-Lindblom, Fischer et al. 2006a). Whilst being biochemically distinct from fungi, Oomycetes have similar morphology, size and habitat usage (Money 1998). In addition, they tend to colonise submerged plant litter in standing water bodies very early in the aquatic decomposition process (Czeczuga, Mazalska et al. 2005; Nechwatal, Wielgoss et al. 2008). It has been suggested that early colonisation may be related to this organism’s inability to hydrolyse cellulose as true fungi can (Dix and Webster 1995). However, research suggests that many species of the Oomycetes possess enzymes that would enable them to degrade cell wall polymers, including cellulose (Latijnhouwers, de Wit et al. 2003; Valikhanov 1988) as well as proteins and lipids (Al-Rekabi, Al-Jubori et al. 2005; Mozaffar and Weete 1993). Fungi are known to compete with bacteria (Mille-Lindblom, Fischer et al. 2006a) and other fungal species (Fryar, Booth et al. 2005) and it is reasonable to assume that Oomycetes are also competing with fungi for resources. These competitive interactions may be particularly important in the relatively harsh conditions that prevail over the summer months (Menge and Sutherland 1987; Nikolcheva and Bärlocher 2004) when stream flow and dissolved oxygen concentrations are reduced.

Fungal biomass accumulates on leaves submerged in rivers (Sridhar and Bärlocher 2000) and standing waters (Mille-Lindblom, Fischer et al. 2006b). Pozo et al. (1998) showed that the leaves of *Eucalyptus globulus* support a similar level of fungal biomass to those of *Alnus glutinosa* in Spanish streams, although on *E. globulus* fungal diversity was lower and colonisation was delayed (Bärlocher and Graça 2002; Graça, Pozo et al. 2002). In Chapter 4 it was found that *E. camaldulensis* leaves in standing water accumulate fungal biomass of similar magnitude to that reported from submerged macrophytes in standing water by Mille-Lindblom et al. (2006b), but this was approximately one tenth of that reported by Pozo et al. (1998). The variation in these results necessitates discrimination between the effects of the leaf substrate and those of streams and standing
waters. Moreover, neither fungal nor Oomycete biomass accumulation, or the influence of this biomass on substrate food quality, has been studied in Australian rivers and floodplain wetlands, and these environments may present unique ecological challenges for aquatic fungi.

In this chapter, I have quantified the fungal and Oomycete biomass that accumulated on *E. camaldulensis* leaves submerged in lowland rivers and floodplain wetlands at three sites over time. I have also examined changes in the structure of the fungal and Oomycete communities and related changes in fungal biomass to changes in community composition, and variations in the nutritional value of the leaf detritus. To achieve this, I devised a field study, where litter bags containing *E. camaldulensis* leaves were submerged in three paired wetland and river sites. The fungal biomass as ergosterol concentration and estimation of hyphal length were compared between the two habitats over 107 days. Fungal and Oomycete community structure were compared over time using Terminal Restriction Fragment Length Polymorphism, and the carbon to nitrogen ratios of partially decomposed leaves were used to estimate their nutritional value in aquatic food webs.

6.2 Methods

6.2.1 Experimental design

Given the differences in substrate quality between fresh and floodplain aged *E. camaldulensis* leaves that were discussed in Chapter 4, only aged leaves were used as substrates in this experiment, in an attempt to reduce variation due to substrate differences. *E. camaldulensis* leaves were collected from the dry floodplain at the Ovens River site and stapled into packs consisting of three leaves then dried at 25°C for a period of 2 weeks in a Contherm Series Five dehydration oven (Contherm Scientific Ltd, Korokoro, New Zealand). Leaf packs were sewn into envelopes made from 500 μm heavy duty nylon mesh with polyester thread. A river rock was included to ensure the leaf packs rested on the surface of the wetland sediment. Sufficient litter bags for all sample times were secured together with nylon fishing line, anchored to a star picket and submerged in 20 – 30cm of water at three positions in each river and wetland site in November 2008. Positions were chosen to correlate with the presence of large river red gums, so that it could be assumed that propagules capable of decomposing these leaves would be present in the environment. A diagrammatic representation of this design is shown in Figure 6.1.
Suitable sites were selected on 3 rivers in north-eastern Victoria. Wetlands were selected because they (a) held water during the experimental period; (b) had close proximity to the stream; and (c) had riparian vegetation dominated by *E. camaldulensis*. Close proximity of the wetland to the stream was intended to equalise factors such as temperature and precipitation, and the influence of soils and vegetation.

Leaf packs were retrieved from river and wetland sites after 2, 15, 30, 60, and 107 days. All samples were removed by 10 March 2009 (Day 107). This sampling pattern was designed to capture temporal changes in the fungal community dynamics and substrate quality, based on results from the mesocosm experiment (Chapter 4). Dry leaves that had not been submerged were denoted as time zero samples. Accumulated sediment was rinsed off litter bags in the water body in which they had been incubated, and then litter bags were stored on ice (~4°C) and transported to the lab where leaves and leaf discs were preserved for further analysis.

### 6.2.2 Field sites

All field sites were situated in north-east Victoria, Australia (Figure 6.2), within the catchment of the Murray River. The catchments of the Ovens and Kiewa Rivers contribute to the larger Murray River catchment with the Kiewa River joining the Murray River upstream of the Ovens confluence. Most rivers in this region are regulated, the Murray River itself having several major impoundments, including the Hume weir at Albury-Wodonga. The exception is the Ovens River, one of the few remaining unregulated rivers in Australia. All sites were used by waterfowl and accessible to cattle.
Sites chosen were:

1. Kiewa River (west bank) at Killara (Vic), and an adjacent wetland (Figure 6.2);
2. Ovens River (east bank), north of Peechelba Rd, Peechelba East (Vic) and an unnamed wetland on its eastern side; and
3. Murray River (north bank), west of Howlong (NSW), and Quat Quatta East Lagoon (see also Chapter 5).

![Field site locations – Victoria, Australia.](image)

**Figure 6.2**: Location of field sites within Australia (inset) and within the state of Victoria.

### 6.2.2.1 Kiewa River, Killara, Victoria

The Kiewa River site and the adjacent wetland were located south of the Murray Valley Highway at Killara, north-east Victoria, Australia (36° 08’ 24.33”S, 146°57’18.37” E, 152 m elevation), within the catchment of the Murray River. The Kiewa River rises in the Victorian Alps and joins the Murray River below the Hume Dam. While it receives hydro-electric power generating discharges from Rocky Valley and Pretty Valley Dams, the flow regime of the Kiewa River is flashy, with low flows in summer, and spates associated with winter/spring rains. Overbank flows associated with winter and spring rainfalls fill the floodplain wetlands. Stream discharge, water level, electrical conductivity and temperature are summarised in Table 6.1. The wetland was full at the
commencement of this study and did not receive any overbank flows throughout the study period, so it was largely dry by the end of the study period. The woodland vegetation was dominated by the river red gum (*E. camaldulensis*) but included some exotic tree species. The surrounding land use was largely pastoral.

![Image](a) ![Image](b)

**Figure 6.3**: The study site on the Kiewa River at Killara showing the wetland (a) and the river (b). River red gums (*E. camaldulensis*) and exotic species dominate the riparian vegetation, and understorey vegetation is dominated by pasture grasses.

### 6.2.2.2 Ovens River, Peechelba East, Victoria

The Ovens River site and the associated wetland are located north of Peechelba Road at Peechelba East, Australia (36°9′ 44.72″S, 146°14′ 16.49″E, 137m), upstream of the Peechelba Flora reserve. The Ovens River rises in the Alps of north-eastern Victoria and has a confluence with the Murray River at Peechelba. It floods seasonally in winter and spring of most years (Quinn, Hillman *et al.* 2000). Stream discharge, water level, electrical conductivity and temperature are summarised in Table 6.1. The wetland, which is filled with flooding flows from a minor creek, was full at the commencement of the study but did not receive any inflows during the study period and was dry by January 19, 2009.

This site was located within the Killawarra State Forest. The vegetation of this area is dominated by *E. camaldulensis* open forest with an understorey consisting of silver wattle (*Acacia dealbata*), river bottlebrush (*Callistemon sieberi*), Chinese scrub (*Cassinia arcuata*), grey parrot-pea (*Dillwynia cinerascens*) and native grasses (*Parks*

(a) (b)

Figure 6.4: The study site on the Ovens River at Peechelba East showing the wetland (a) and the river (b). River red gums (*E. camaldulensis*) dominate the riparian vegetation, and understory vegetation is dominated by native grasses.

6.2.2.3 Murray River, Howlong, New South Wales

The Murray River site was located west of Howlong, New South Wales, at the Point where Quat Quatta East Lagoon connects with the river (35° 58’ 19.82” S, 146° 34’ 44.67” E, Elevation 150m elevation). The Murray River is one of Australia’s longest rivers, having its source in the Snowy Mountains of New South Wales and draining west into Lake Alexandrina in South Australia. It is highly regulated (Maheshwari, Walker *et al.* 1995), supplying water for irrigation, stock usage and domestic supply over the warmer months, while flows in cooler months are retained in storages. The water level in Quat Quatta East lagoon decreased until the wetland was nearly dry during the study period, but it received inflows via an inlet from the Murray River in late December 2008, and began to refill. Stream discharge, water level, electrical conductivity and temperature for the Murray River downstream of the study site at Corowa (36° 0’ 25.92” S, 146° 23’ 43.08” E) are summarised in Table 6.1. The vegetation around the Murray River and Quat Quatta East Lagoon is dominated by *E. camaldulensis* open forest with a grassy understory (Margules and Partners Pty Ltd, P and J Smith Ecological Consultants *et al.* 1990; Pressey 1986). The area is grazed by cattle and used for camping and recreational fishing.
Figure 6.5: The Murray study site showing Quat Quatta East lagoon (a) and the Murray River west of Howlong (b). River red gums (*E. camaldulensis*) dominate the riparian vegetation along this stretch of the river and around wetlands, and understorey vegetation is dominated by grasses.

Table 6.1: Summary of the environmental conditions in the river at each site over the study period. Data are based on the mean value of mean daily readings, with maximum and minimum mean daily readings going to comprise the stated range. Data were obtained from the Murray-Darling Basin Authority website (http://www.mdba.gov.au/water/live-river-data).

<table>
<thead>
<tr>
<th>Location</th>
<th>Discharge (ML/day)</th>
<th>Electrical Conductivity (µS.cm⁻¹)</th>
<th>Water Level (m)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovens River at Peechelba</td>
<td>507</td>
<td>55.83</td>
<td>1.17</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>(106-1975)</td>
<td>(21.07-82.11)</td>
<td>(0.54-3.04)</td>
<td>(18.2-30.4)</td>
</tr>
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<td>Murray River at Corowa</td>
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<td>2.12</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>(5076-11910)</td>
<td>(38.67-64.59)</td>
<td>(1.49-2.58)</td>
<td>(17.7-28.3)</td>
</tr>
<tr>
<td>Kiewa River at Killara</td>
<td>535</td>
<td>33.54</td>
<td>0.94</td>
<td>22.1</td>
</tr>
<tr>
<td></td>
<td>(109-2421)</td>
<td>(16.98-58.93)</td>
<td>(0.58-2.05)</td>
<td>(4.1-29.6)</td>
</tr>
</tbody>
</table>

6.2.3 Organic matter content

The leaf mass loss on ignition was determined in the same manner as described earlier in 4.2.2.

Data for mass loss and change in organic matter content were analysed using two-way analysis of variance (ANOVA) with replication (3) for each sampling time. Time was used as the covariant, pair-wise for treatments (Bärlocher 2005). Data analyses were performed using SPSS Statistics 17.0 (IBM, Chicago, USA).
6.2.4 Carbon to nitrogen ratio

6.2.4.1 Carbon content

The carbon content of leaves was estimated as 50% of the ash-free dry mass (Lindroth, Kopper et al. 2001), and expressed as mgC.cm$^{-2}$ leaf area.

6.2.4.2 Total nitrogen content

Leaf samples taken from litter bags were stored frozen and then dried at 80°C in a dehydration oven for 4 days. Leaves were then ground to a coarse powder in a coffee grinder and 5 - 50mg of leaf material was then weighed into a 30ml vial. Samples were then analysed for Total Nitrogen Content (TPN) at the analytical chemistry lab of the Murray-Darling Freshwater Research Centre, Wodonga, following sodium hydroxide / potassium persulfate digestion and conversion to the diazonium salt (American Public Health Association, American Water Works Association et al. 1989).

Leaf mass was converted to leaf area using a conversion factor derived from the Dry Mass determination of leaf discs from each site and at each time. Nitrogen content was then expressed as mgN.cm$^{-2}$ leaf area.

6.2.4.3 Mean carbon to nitrogen ratio

The carbon to nitrogen ratio was calculated by dividing mean leaf carbon content by mean leaf nitrogen content, where both parameters were expressed in mg.cm$^{-2}$.

6.2.5 Fungal biomass

6.2.5.1 Ergosterol content

Fungal biomass was calculated from the ergosterol content of partially decomposed leaves. The method used to quantify ergosterol concentration and calculate fungal biomass has been detailed in Section 4.2.3. However, in this instance, leaf discs were 9mm in diameter, and not 12mm, and HPLC run time was reduced to 14 minutes, from 20 minutes.

6.2.5.2 Hyphal bio-volume

The biomass of filamentous fungi and stramenopiles on and within leaf discs were determined using the “Membrane filtration method” (Hanssen, Thingstad et al. 1974; Robinson, Borisova et al. 1996), which uses fluorescent dyes to simplify counting.
Briefly, three leaf discs (9mm in diameter) from each site replicate at each time were preserved in 10ml of 1M potassium hydroxide (KOH) and stored at 4°C. Replicate leaf discs were weighed, and then dried at 105°C for 1 week before being re-weighed to determine the fresh: dry mass ratio for the leaf material.

Prior to analysis, the discs in 1M KOH were heated to 100°C in a heating block for 15 minutes. Then the KOH was drained off and the discs were washed three times in 10ml of distilled water for 15 minutes. This process clears the leaf material and improves contrast between fungal material and the leaf matrix (Díez-Navajas, Greif 

The three leaf discs in each sample were then blotted dry and weighed, with the mass recorded to 4 decimal places. These discs were then homogenised in a tissue grinder and suspended in 50 ml of 67mM K$_2$HPO$_4$ (phosphate buffer, pH 9.0). This suspension was then further diluted (1:5) with phosphate buffer. Five ml of this diluted sample, mixed with 2 drops of 0.1% phenolic aniline blue, was then filtered through a black polycarbonate, 25mm membrane filter (0.8µm pore size) (GE Osmonics, Minnetonka, MN, USA). Three replicate filters were prepared for each suspension, and these were blotted dry and mounted on a standard glass slide in “Cargille” (non-drying, low UV fluorescence) immersion oil Type B (ProSciTech, Thuringowa, Qld). Slides were examined under a Zeiss Axioskop2 epifluorescence microscope (Ziess, Oberkochen, West Germany) at 200 x magnification using a Nikon UV-1A filter cube.

Hyphal length estimates were made with an eyepiece grid at five randomly selected positions on the filter using the grid-intersection method (method 5) as reported by Olson (1950). The diameters of all filaments in one grid of each filter were measured, and mean hyphal diameter was then calculated. Mycelial biomass was calculated from hyphal length following the method proposed by Frankland et al. (1978). These authors used the following equation: $B = \pi r^2 \times L \times D \times M$ (where $B$ is the mass of oven dried mycelium in oven dried litter (g.g$^{-1}$); $r$ is hyphal radius in mm; $L$ is hyphal length in meters per mg of oven dried litter; $D$ is the relative density of the hyphae (g.cm$^{-3}$); and $M$ is the percentage moisture content of fresh hyphae divided by 100). Literature values for hyphal density range from 1.1-1.5 g.cm$^{-3}$ (Van Veen and Paul 1979) and moisture content generally ranges from 80-90% (Hawker, 1950). I used an estimated density of 1.2 g.cm$^{-3}$ and an estimated moisture content of 85%.
Fungal biovolume data were analysed using 3-way ANOVA on the SPSS software package (IGM, Chicago, USA), analysing between leaf type, treatments and times ($\alpha=0.05$). The fungal biomass results from ergosterol and hyphal biovolume analyses were standardised by dividing each value by the total of all values obtained for each method and compared using simple linear regression (SPSS, IGM, Chicago, USA).

6.2.6 Fungal community structure

6.2.6.1 Total fungal community
Fungal community structure was assessed using terminal restriction fragment length polymorphism (T-RFLP). This method is described in Section 4.2.5.

6.2.6.2 Oomycetes community structure
Amplification of the ITS region of the Oomycetes DNA was carried using the same genomic DNA template as the total fungal community. Amplification of Oomycete DNA followed the procedure reported by Nikolcheva and Bärlocher, (2004), and differed from the method described in Section 4.2.5 in terms of the length of DNA amplified, the primers used, and the thermocycler protocol. Briefly, primers used amplified the entire ITS region, extending from 18S rDNA to 28S rDNA. The forward primer was ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG–3') (White, Bruns et al. 1990), which carried a fluorescent label (5,6 Carboxyfluorecein) at the 5' end, and the reverse primer was ITSOo (5'-ATAAGACTACAATCGCC – 3') (Nikolcheva and Bärlocher 2004). Primers were synthesised by Gene Works (Hindmarsh, South Australia). These primers produce DNA fragments that were approximately 1.25 kb in length and were specific to Oomycetes.

Amplification was carried out in an “iCycler” (Bio-Rad, Hercules, CA, USA) thermocycler. The protocol consisted of initial denaturation at 95°C for 15 minutes (1 cycle), then 35 cycles of denaturation at 94°C for 15 seconds, annealing at 49°C for 15 seconds, and extension at 72°C for 60 seconds. A final cycle was included with denaturation at 94°C for 15 seconds, annealing at 49°C for 90 seconds, and extension at 72°C for 6 minutes. The PCR product was then held at 4°C until being removed from the thermocycler and stored at -20°C.
6.3 Results

6.3.1 Mass loss and organic matter content

6.3.1.1 Organic matter content

3-way ANOVA on leaf organic matter content data showed that organic matter content differed significantly between rivers and wetlands (P<0.005) and over time (P<0.05). These results are shown in Figure 6.6. No significant difference was found between the Ovens, Murray and Kiewa river systems (P=0.649) or with interaction effects. The general pattern for all sites was for a rapid decrease in organic matter content over the first 15-30 days. Following this, organic matter content did not change and was consistently lower in leaves from rivers than in those from wetlands.

6.3.2 Nitrogen content

3-way ANOVA showed that nitrogen content of submerged leaves differed significantly between sample times (P<0.005), but not between sites or between rivers and wetlands (P= 0.397 and P=0.258, respectively). However, there was an interaction effect between river systems and time (P= 0.054). While increases in nitrogen content were seen on some days in some sites, the nitrogen content generally decreased over time.

The nitrogen content of submerged leaves changed over time, and these results are illustrated in Figure 6.7. In the Ovens system mean increases of 49 and 47% nitrogen content were seen after 2 days in the river and the wetland, respectively, although there was large variation between replicates. By day 15, nitrogen content was 40% lower than the initial value in the wetland, but remained similar to initial levels in the river. The nitrogen content of leaves submerged in the Ovens system was approximately half of the initial value for the remainder of the incubation period, mean values increased with time but variation between replicates was high. In the Murray system nitrogen content decreased by approximately 20-25% between the initial value and the value after 2 days submerged. Following this, small decreases were seen over time, with little difference between the river and wetland samples, except at 30 days submerged. At this time, leaves in the river had mean nitrogen contents 75% higher than those in the wetland. In the Kiewa system mean nitrogen content decreased by 30-50% between day 0 and day 15.
and then remained constant at approximately 50% of the initial value, while mean nitrogen content decreased slightly at day 107.
Figure 6.6: Change in the mean organic matter of leaf packs over a 107 day incubation period is shown for the Ovens (i), Murray (ii) and Kiewa (iii) sites. Samples incubated in the river indicated by open symbols while samples from wetlands and indicated by solid symbols. The x-axis shows time in days, while the y-axis shows the organic matter content of leaf discs as grams per square centimetre. Error bars indicate the standard error of the data point.
Figure 6.7: The nitrogen content of leaves taken from the Ovens (i), Murray (ii) and Kiewa (iii) sites. Samples incubated in the river indicated by open symbols while samples from wetlands and
indicated by solid symbols. Ovens samples are shown as circles, Murray samples as triangles, and Kiewa samples as diamonds. The x-axis shows time in days, and the y-axis shows nitrogen content in mg.cm$^{-2}$. Error bars show standard error of the mean of each data point.

6.3.3 Mean carbon to nitrogen ratio

The data obtained from leaves in litter bags (Figure 6.8) indicate that C:N tends to decrease in leaves in rivers over the first 15-30 days of aquatic incubation. After this time, leaf packs had C:N values close to that of floodplain aged (Day 0) leaves. Leaves incubated in the wetlands showed decreases in C:N after 2 days. Over days 15-30, leaves taken from the Ovens and Kiewa wetlands showed C:N above that of floodplain aged leaves, but by 60 days C:N was again similar to floodplain aged leaves. Leaves incubated in the Murray wetland showed similar trajectory of change in C:N to river samples from the same sample time, although C:N was generally lower in the river samples.

3-way ANOVA of C:N indicated differences between sites (P=0.067) and over time (P=0.055), but these differences were not significant at the 95% confidence level. A significant interaction effect was detected between site and time (P<0.005).

The carbon: nitrogen ratio (C:N) results are shown in Figure 6.8 compared with the C:N of 29:1. C:N values below this figure have been reported to represent a nutritious food source for invertebrates (Esteves and Barbieri 1983). Submerged $E. \text{camaldulensis}$ leaves exceeded a C:N of 29:1 in the first 1-2 months after inundation. Sample times when C:N fell below 29:1 varied between sites, and between rivers and wetlands. In the Ovens system submerged $E. \text{camaldulensis}$ leaves showed C:N below 29:1 after 2 days, but by 15 days leaves submerged in the wetland had C:N above this value and this persisted for the remainder of the incubation period. After 15 days submerged leaves from the Ovens River had C:N below the 29:1 but by 30 days C:N was similar to leaves from the wetland. In the Murray system the C:N value was below 29:1 at 15 and 30 days aquatic incubation, but rose above this value after 60 day. The C:N was always above 29:1 in the Kiewa wetland, but in the Kiewa River C:N was less than 29:1 at sample day 2, and approximately equal to 29:1 at day 107.
Figure 6.8: Carbon to nitrogen ratio of leaf litter from litter bags in the river and wetland at the Ovens (i), Murray (ii) and Kiewa (iii) sites. The x-axis shows the incubation time in days, while the y-axis indicates the ratio of mean carbon content (mgC.cm$^{-2}$) to mean nitrogen content (mgN.cm$^{-2}$). Wetland sites are indicated by solid symbols, river sites are indicated by open symbols. The red line
indicates the approximate value below which a resource is considered nutritious to invertebrates. At day 30 C:N in the Ovens River and wetland were similar.
6.3.4 Fungal biomass

6.3.4.1 Ergosterol content

Analysis of ergosterol content data indicated that fungal biomass differed significantly over time (3-way ANOVA: P<0.005), and differed between rivers and wetlands (3-way ANOVA; P=0.051), but no significant difference was found between river systems, or with interaction effects. No significant differences were found using pair-wise t-tests to compare biomass in river and wetlands and across sites.

When considered on an areal basis, biomass tended to increase over 15-30 days then decrease. The time when maximum mean fungal biomass was observed differed between sites. Maximum biomass occurred after 30 days in the Kiewa River, 15 days in the Ovens River and 2 days in the Murray River (Figure 6.9). In the wetlands maxima occurred after 2 (Kiewa), and 15 (Ovens and Murray) days. Fungal biomass decreased thereafter, although rates of decrease differed. While fungal biomass tended to be higher in the river than in the wetland at the Murray and Kiewa sites, at the Ovens site biomass was higher in the wetland.

Linear regression indicated no direct relationship between leaf nitrogen content and fungal biomass as estimated by ergosterol content (r²=0.010; P<0.05) or hyphal length (r²=0.052; P<0.05).

6.3.4.2 Hyphal biovolume

Fungal biomass as determined by hyphal biovolume estimation differed from estimates by ergosterol concentration. Maximum mean fungal biomass was seen in pre-inundation leaves at all sites, decreasing with time (Figure 6.9) and differing significantly between times (3-way ANOVA: P<0.005). Contrary to results from the analysis of ergosterol concentration data, no difference was found between sites or between rivers and wetlands (3-way ANOVA: P>0.05). Some correlation was found between fungal biomass as measured by ergosterol and hyphal biovolume (Regression: r² = 0.57; P<0.005), but a large amount of variation in hyphal biovolume data was not explained by ergosterol content data.
In general, rivers and wetlands at each site showed similar patterns of temporal change, with lower mean biomass in wetlands than in rivers, although the difference was not significant. This pattern is seen most clearly at the Ovens and Murray sites. At the Kiewa site an increase in fungal biomass was seen in the wetland after 60 days, and at this time fungal biomass in the wetland exceeded that in the river. However, there was no significant difference in biomass (by biovolume) between sites for this time (3-way ANOVA: P=0.302).

6.3.4.3 **Biomass estimates by ergosterol content compared with hyphal biovolume**

Fungal biomass values measured as ergosterol content generally decreased over time, while the combined biomass of fungi and Oomycetes, as measured by hyphal biovolume, showed initial decreases then remained stable. The difference between fungal biomass estimated by ergosterol and hyphal length suggests that the biomass of the Oomycetes was more substantial during the first few days of aquatic decomposition, and after 60 days.

The differences in the two fungal biomass data sets (ergosterol and hyphal biovolume) resulted from differences in magnitude and in the direction of change over time. Firstly, estimates of biomass by hyphal biovolume were generally higher than those estimated by ergosterol content, when data were expressed per area of leaf. Secondly, while ergosterol analysis indicated decreasing fungal biomass after a maximum at 2-15 days (30 days in the Kiewa River), hyphal biovolume data showed that biomass decreased for 15-30 days after inundation, then remained stable or increased.
Figure 6.9: Fungal biomass from leaves submerged at the Ovens (i), Murray (ii) and Kiewa (iii) sites. Open symbols indicate river samples while solid symbols indicate wetland samples. Samples at the Ovens sites are represented by circles, samples at the Murray site are represented by triangles and samples at the Kiewa site are represented by diamonds. Un-dotted (white and black) symbols...
indicate biomass determined by biovolume, while dotted symbols (White and grey) indicate biomass measured by ergosterol concentration. Error bars indicate standard error about the mean at each data point.

6.3.5 Community structure

6.3.5.1 Total fungal community

Analysis of all T-RFLP data indicates that the fungal community differed with time (ANOSIM: R=0.550; P<0.005; see also Figure 6.10), but not between sites, or between rivers and wetlands. Pair-wise ANOSIM showed that community structure was similar between subsequent sampling days (R<0.48; P<0.419). However, fungal community structure at day 30 was significantly different to the community structure prior of immersion in the water (R=0.809; P<0.05), and after 107 days in the water (R=0.683; P<0.005). Fungal community structure was also significantly different between Day 2 and Day 107 (R=0.754; P<0.005). The trajectory of change with time (Figure 6.11) has 2 directions. For river sites, diversity increases between days 2 and 30 then decreases. In wetlands, changes in the number of amplicons have no apparent pattern.

When T-RFLP data from the three sites were considered independently, the river and wetland differed at the Kiewa site (R=0.542; P<0.005) (Figure 6.12), but were similar at the Ovens (R=0.075; P=0.668) (Figure 6.13) and Murray (R=0.036; P=0.469) (Figure 6.14) sites. The fungal community structure was similar between sites when rivers (R=0.091; P=0.197) and wetlands (R=0.353; P<0.005) were considered independently and pair-wise tests showed no significant differences between river sites (R<0.145; P>0.135). However, the Kiewa wetland differed somewhat from the Ovens (R=0.364; P<0.05) and Murray (R=0.532; P<0.05) wetlands, while the Ovens and Murray wetlands were similar (R=0.139; P=0.267) (Figure 6.15). When data were analysed separately for each sampling occasion, wetland sites and river sites (R=0.596; P<0.05) and river systems (R=0.600; P<0.005) can be distinguished on Day 60 (Figure 6.16). A sample from the Ovens River was not clustered with other river samples. These leaves may have been periodically exposed due to low flows in the river.
Figure 6.10: MDS ordination plot of T-RFLP results showing fungal community structure changes over time. Hexagons as shown in the legend indicate community structure on leaves prior to being submerged (Day 0), and after 2, 15, 30, 60 and 107 days submerged in the water. Circles indicate the grouping of samples from 30 days and before on the left, and from 60 days and after on the right. X- and Y-axes indicate relative position in multivariate space and have no units.

Figure 6.11: This plot indicates the mean position of MDS points from each sampling occasion, with error bars indicating standard error about that mean. The data indicate that fungal community structure changed significantly between Day 0 and day 30, then changed significantly in a different manner between day 30 and Day 107. X- and Y-axes indicate relative position in multivariate space and have no units.
Figure 6.12: An MDS plot of T-RFLP from the Kiewa System showing variation in fungal community structure between river and wetland sites at days 30, 60 and 107, but not at earlier times. X- and Y-axes indicate relative position in multivariate space and have no units. Circles indicate samples taken at the same time and have no statistical meaning. Numbers indicate sample time for individual data points.

Figure 6.13: An MDS plot of T-RFLP from the Ovens System showing only small differences in fungal community structure between river and wetland sites within sampling times. X- and Y-axes
indicate relative position in multivariate space and have no units. Circles indicate samples taken at the same time and have no statistical meaning.

Figure 6.14: An MDS plot of T-RFLP from the Murray System showing small differences in fungal community structure between river and wetland sites within sample times, although the ordination will be influenced by the small number of replicates for Murray wetland samples. X- and Y-axes indicate relative position in multivariate space and have no units. Numbers indicate sample time (days).
Figure 6.15: An MDS plot of T-RFLP data from the wetland samples showing differences in fungal community structure between wetland sites and clustering by sampling time. Circles are intended to indicate groupings by time and do not indicate a statistical difference. X- and Y-axes indicate relative position in multivariate space and have no units. Numbers indicate sample time (days).

Figure 6.16: An MDS plot of T-RFLP from the Day 60 showing that the fungal community structure tends to cluster by river and wetland sites, and wetland also cluster by river system. Circles are
intended to indicate groupings by time and do not indicate a statistical difference. X- and Y-axes indicate relative position in multivariate space and have no units. An outlying sample from the Ovens River may have been periodically exposed due to low flows in the river.

6.3.5.2 Oomycete community structure

PCR of genomic DNA from time zero (floodplain aged) samples did not produce any product DNA. This may indicate that Oomycetes were absent from these samples, or that inhibiting substances in these samples interfered with the amplification reaction. Thus, time zero samples were excluded from data analysis rather than being given a zero value.

Ordination of T-RFLP results showed that the Oomycetes community changed over time (Figure 6.17). This was a gradual transition, with substantial overlap between data from different times. Thus, separation was not significant when all data were considered (R=0.344; P<0.005). However, pair-wise tests showed that dissimilarity between day 2 and subsequent days increased from day 15 (R=0.235; P<0.005), through to day 30 (R=0.436; P<0.005), day 60 (R=0.569; P<0.005) and day 107 (R=0.844; P<0.005). There is a clear trajectory for this change (Figure 6.18) which is driven by increasing numbers of taxonomic units, although the pattern of this change differs between sites. At the Ovens site the number of T-RF lengths increased until day 15 in the wetland, and day 30 in the river, and then declined. At the Murray site, the number of different T-RF lengths increased steadily over time, and at the Kiewa sites this number fell until day 30, and then rose again.
Figure 6.17: MDS ordination plot showing Oomycete community structure with time. Hexagons as shown in the legend indicate community structure on leaves after 2, 15, 30, 60 and 107 days submerged in the water. X- and Y-axes indicate relative position in multivariate space and have no units.

Figure 6.18: The mean position of all points from each time as shown in Figure 6.17. The axes represent dimensions of ordination space and have no labels. Error bars indicate the standard error of the data point. This graph shows a clear change in the composition of the Oomycete community moving from left to right, driven by increases in the number of T-RF lengths.
Figure 6.19: MDS ordination plot of the Oomycete community structure showing a low degree of separation between sites, and between rivers and wetlands. River sites are indicated by open symbols while wetland sites are indicated by solid symbols. Symbols indicate the Kiewa (diamonds), Murray (triangles) and Ovens (circles) sites.

Ordination of Oomycete T-RFLP data showed that the degree of separation between river systems was low (Figure 6.19) (ANOSIM: R=0.25; P<0.005), and pair-wise tests showed no significant separation between individual sites. When data were analysed separately for wetlands, the separation between sites improved (R=0.451; P<0.005). River sites were more dissimilar (R=0.603; P<0.005), with pair-wise tests showing that this result was largely driven by differences between the Murray and Kiewa Rivers (R=0.674; P<0.005).

The degree of dissimilarity among sites changed with time. Separation between sites was lowest at day 2 (R=0.289; P=0.052), reached a maximum at day 15 (R=0.738; P<0.05), decreased again at day 30 (R=0.401; P<0.05), then increased at day 60 (R=0.59; P<0.05). At day 15, wetland samples were separated by site (Figure 6.20), but river samples clustered into a single group. Pair-wise analysis showed that all sites were dissimilar (Kiewa/Ovens R=0.701; Kiewa/Murray R=0.822; Ovens/Murray R=0.688; P<0.05).

While some separation between river and wetland samples was discernable, ANOSIM (R=0.28; P<0.05) showed that separation was low for rivers and wetlands when all sites and times were considered. When a single site was considered, ANOSIM showed greater dissimilarity between rivers and wetlands in the Murray (R=0.573; P<0.005) and Kiewa systems (R=0.62; P<0.005) (Figure 6.21).
Figure 6.20: MDS ordination plot of Oomycete T-RFLP data from 15 days submerged leaf packs. River sites are indicated by open symbols while wetland sites are indicated by solid symbols. Symbols indicate the Kiewa (diamonds), Murray (triangles) and Ovens (circles) sites. Circles are drawn to illustrate groupings and do not imply statistical similarity.
6.4 Discussion

This study has shown that the food quality of submerged leaves and the biomass and community structure of fungi and Oomycetes changes over time in the aquatic environment. Food quality indicators and fungal biomass (ergosterol) generally decreased over time, while the combined biomass of fungi and Oomycetes (hyphal biovolume estimates) showed initial decreases then remained stable. The diversity of Oomycetes also increased over 107 days in the aquatic environment. Changes observed in the structure of the fungal and Oomycete communities, biomass and leaf resource quality are consistent with succession in the decomposer community as resources within the leaf are consumed, altering its chemical composition.

6.4.1 The nutritional value of submerged *E. camaldulensis* leaves

Fresh *E. camaldulensis* leaves have been reported to have a high nitrogen content of 1.29% dry weight (Cork and Pahl 1996; Taylor, Parkinson *et al.* 1989), and this work shows that floodplain aged leaves have a similar nitrogen content (1.28 ± 0.60% dry weight). This suggests that while nitrogen is undoubtedly being used by terrestrial microorganisms, most is being retained within the leaf. This nitrogen is likely to be either in the form of microbial biomass or complexed with lignin. These results do not imply that there has been no loss of nitrogen, but that relatively more carbon has been lost through processes such as respiration. The relative loss of carbon is illustrated by changes in C:N with litter decomposition. While surface litter has a C:N of around 100:1, the ratio falls to 30-15:1 in partially decomposed litter and 10:1 in humus (Griffin 1972). This study shows that when floodplain aged *E. camaldulensis* leaves were inundated the C:N decreased, and the change in C:N was driven by decreases in organic matter content and short-term increases nitrogen content. However, lower C:N values were limited to the first 30 days after inundation, and the pattern of change differed between sites.

The C:N of a food resource is an indication of its food value to consumer organisms (Boyd and Goodyear 1971; Cummins and Klug 1979; Hladyz, Gessner *et al.* 2009), and
C:N of 29:1 has been nominated as the nutrient balance suitable for invertebrate consumers (Esteves and Barbieri 1983). A previous study (Glazebrook and Robertson 1999) indicated that floodplain aged *E. camaldulensis* leaves had a C:N of 95:1, and when these leaves were inundated on the floodplain C:N fell to 45:1 after 49 days; subsequently C:N increased to 132:1 after 112 days. The current study has further shown that this increase in food value is temporary and site specific, with C:N being lower than 29:1 for 2 days in the Kiewa River and Ovens wetland, 15 days in the Ovens River and 30 days in the Murray River and the associated wetland. C:N on leaves in the Kiewa wetland was never less than 29:1. As the substrate was the same in all cases, these differences in C:N are likely to be driven by environmental factors such as nutrient concentrations. Change in C:N as leaves are inundated could also be expected to differ between tree species (Kominoski, Hoellein *et al.* 2009). After 60 days of inundation, C:N values were similar to those found by Glazebrook and Robertson (1999) after 49 days.

Inundated *E. camaldulensis* leaves break down quickly relative to the leaves of other eucalypt species (Schulze and Walker 1997), and leaching that occurs during the first 30 days in the water has a strong influence on breakdown rates (Janssen and Walker 1999; Liakos 1986; O’Connell, Baldwin *et al.* 2000). Fast decomposition rates have previously been associated with low C:N values (Enriquez, Duarte *et al.* 1993), but this study has shown low C:N values are found in *E. camaldulensis* leaves for a limited period. This period corresponds with maximum organic matter losses due to leaching, and differs between rivers and wetlands.

Periods when loss of organic matter from submerged leaves was most rapid also corresponded with periods of higher fungal biomass. Negligible changes in organic matter content after 30 days of inundation similarly corresponded to lower fungal biomass. This pattern was consistent with carbon consumption and respiration by the decomposer community, but the change in biomass may indicate a response by fungal decomposers to the decreased availability of specific, readily metabolised leaf components, rather than organic matter generally. Furthermore, microbial activity and leaching interact during aquatic litter decomposition (Bunn, De Deckker *et al.* 1986; Campbell, James *et al.* 1992b), so the contribution of leaching, respiration and fungal reproduction to losses of organic matter from *E. camaldulensis* leaves should be explored further (cf. Pascoal and Cassio 2004).
The nitrogen content of floodplain aged *E. camaldulensis* leaves was 0.045 mg.cm\(^{-2}\) and reached a maximum value of 0.067 mg.cm\(^{-2}\) after 2 days submerged in the Ovens River. This suggests low levels of nitrogen incorporation into *E. camaldulensis* leaves. This period of nitrogen enrichment during the early phase of aquatic decomposition corresponded with periods of high estimated Oomycetes biomass (see section 6.4.2). As these organisms are known to be able to enrich their substrates with nitrogen (Willoughby and Redhead 1973), they may be important in improving the nutritional value of submerged eucalypt leaves. It should be possible to test this hypothesis using nitrogen isotopes to trace nitrogen from the water column through to its incorporation into proteins in leaves colonised by Oomycetes and aquatic hyphomycetes.

Carbon and nitrogen in fungal biomass are generally found in the ratio of 12-8:1, and the optimum C:N for an artificial fungal substrate has been reported to be around 15:1 (Griffin 1972). A substrate C:N above 40:1 can inhibit the production of conidia (Jackson and Bothast 1990), and a substrate with C:N >30:1 would be considered to be nitrogen limited (Melillo, Aber *et al.* 1982). As the floodplain aged leaves of *E. camaldulensis* have a C:N of around 40:1, they could be considered to be a nitrogen limited substrate. On such substrates, biomass accumulation may be limited (Stelzer, Heffernan *et al.* 2003) and fungi may recycle nitrogen internally in order to maintain growth by extending hyphal tips (Boswell, Jacobs *et al.* 2002; Cowling and Merrill 1966; Paustian and Schnurer 1987). Under these circumstances fungi will allocate most of their nitrogen to cell wall synthesis (Paustian and Schnurer 1987). This would result in increased fungal biomass measurements, whether estimated by ergosterol content or hyphal length, with no corresponding net increase in the nitrogen content of the substrate/mycelial complex. Thus, in the absence of a bioavailable external nitrogen source fungal colonisation of submerged *E. camaldulensis* leaves may not result in reduced C:N or increased nutritional value. Nitrogen limitation may be significant in the role of fungi in aquatic carbon cycles as low nitrogen availability is known to stimulate lignin degradation by fungi (Kirk and Farrell 1987).

Over the experimental period, total nitrogen in the water column was highest in the Murray River and lowest in the Kiewa River (0.6mg/L and 0.2mg/L, respectively, in March) (Victorian State Government, EPA Victoria *et al.* 2010). This represents
moderate to high levels of dissolved nitrogen. While aquatic fungi are capable of utilising inorganic nitrogen from the water column (Suberkropp and Chauvet 1995), nitrogen enrichment may stimulate production by benthic and pelagic algae, and fungi may then utilise algal proteins as a nitrogen source (Artigas Alejo 2008). Either way, higher ambient nitrogen in the Murray system is correlated with lower C:N, and conversely lower ambient nitrogen in the Kiewa system is correlated with higher C:N ratios. In support of this notion, the C:N of aged *E. delagatensis* submerged in a nitrogen limited upland stream did not record values lower than 29:1 over a 64 day incubation period (Love 2005).

6.4.2 **Fungal and Oomycete biomass**

The differences in the timing of fungal biomass maxima between sites and in the trajectory of change in biomass in the two fungal biomass data sets (ergosterol and hyphal biovolume) may be related to the flow regime. At day 60 conditions were hot, the Ovens and Kiewa wetlands were drying and flows in these rivers were low. Low flows may encourage increased Oomycete biomass and discourage increases in eumycota biomass. At the same time the Murray River had high flow rates (due to irrigation flows) and the wetland was receiving inflows. At this site, there was no suggestion of a significant increase in Oomycete biomass.

Ergosterol concentration reflects the biomass of filamentous fungi and yeasts (Eumycota) (Gessner and Chauvet 1993), including spores, conidia and vegetative cells in addition to the growing mycelium (Newell 1992). Biomass estimated by biovolume (hyphal length) reflects the abundance of Oomycetes (Stramenopila) as well as filamentous fungi, but excludes yeasts, spores, conidia and non-filamentous fungi. Therefore, significant differences in biomass between rivers and wetlands by ergosterol concentration, but not by hyphal length determination are not inconsistent. Together these data suggest that differences between rivers and wetlands were driven by the abundance of true fungi (Eumycota), and that the combined biomass of Eumycota and Oomycota did not vary spatially.

Aquatic hyphomycetes invest a significant proportion of their biomass into the production of conidia which are subsequently released into the water and lost from the decaying leaves (Suberkropp 1991b). The highest rates of sporulation occur at the end of
the growth phase, and this has been shown to occur after approximately 18 days when leaves are submerged in a stream (Suberkropp, Gessner et al. 1993). Fungal biomass on submerged leaves increased for 15 days in the Ovens River and wetland, Murray wetland and Kiewa River using the ergosterol method. These increases were not reflected in the biovolume data. This difference probably represents the production of conidia because these propagules contain ergosterol but are not included in biovolume estimates. Subsequent declines in biomass were consistent with the loss of fungal biomass due to sporulation. Gradual decreases in biomass in the Kiewa wetland and Murray River are consistent with the gradual loss of mycelial tissue, and may indicate the absence of a reproductive event.

Biomass estimated by biovolume indicated an increase in mycelial mass after 60 days in the Ovens River and wetland, and after 30-60 days in the Kiewa River and wetland. These increases were not reflected in ergosterol data. Thus, this increase probably indicates increasing Oomycete biomass as flows in the rivers decreased and water levels in the wetlands declined. This vegetative growth event was absent from the Murray site, where flows were increasing in the river and the wetland was receiving inflows. Biomass estimated from biovolume was also relatively high on Day 0 and Day 2. This would also indicate a significant proportion of the biomass was composed of Oomycetes, and is consistent with literature suggesting they are common during the early stages of aquatic decomposition (Bärlocher 1990; Bärlocher and Kendrick 1974), and in terrestrial plants and litter (Neher 1999). Biovolume biomass is consistently higher than ergosterol biomass during the later phase of aquatic decomposition. This may be due to the presence of non-living hyphae which would lose their ergosterol content soon after death but remain visible by epifluorescence microscopy.

Pozo et al. (1998) found that the leaves of *Eucalyptus globulus* (blue gum) support similar amounts of fungal biomass to *Alnus* sp. (alder). The maximum fungal biomass found on *E. camaldulensis* leaves in this study was 3.69% of AFDM, while maximum biomass reported for *Alnus* sp. ranged from 5.8-9.8% AFDM (Ferreira, Gulis et al. 2006; Gessner and Chauvet 1994; Gessner, Robinson et al. 1998; Hieber and Gessner 2002) and for *Salix* sp. ~8% AFDM (Baldy, Gessner et al. 1995; Hieber and Gessner 2002). This indicates that the fungal biomass found on *E. camaldulensis* leaves in Australian
streams and wetlands in this study was approximately half of that seen on deciduous tree leaves in streams and wetlands of temperate zones of the northern hemisphere.

Possible causes of lower fungal biomass on eucalypt leaves in Australian systems include substrate quality, and seasonal and site differences. The chemical composition of the freshly abscised *E. globulus* leaves used by Graça *et al.* (2002) will have been different to the floodplain aged *E. camaldulensis* leaves used in this study because *Eucalyptus* species differ in respect to concentrations of nutrients (Cork and Pahl 1996), essential oils (Gilles, Zhao *et al.* 2010), and anatomical features (Pereira and Kozlowski 1976), while floodplain aging is also known to alter the chemical character of eucalypt leaves (Baldwin 1999). Season is known to influence fungal biomass, with higher biomass reported in winter (8.8% detrital mass) than in summer (4.4% detrital mass) (Suberkropp 1997) in a stream at a similar latitude to those examined in this study. However, in higher latitudes, peak fungal biomass on submerged litter occurs in both spring and autumn (Verma, Robarts *et al.* 2003). The values reported from this study in the Murray River system over the summer months fall within the range of summer biomass results reported by Suberkropp (1997). Site differences such as flow rate, the concentration of dissolved oxygen (Charcosset and Chauvet 2001), the probability of burial due to sedimentation (Cornut, Elger *et al.* 2010) and the concentration of nutrients and inhibiting substances in the water column (Stelzer, Heffernan *et al.* 2003; Suberkropp and Chauvet 1995) will also affect fungal biomass accumulation. The abundance of Oomycetes has also been reported to vary seasonally (Hallett and Dick 1981), with maxima in early spring, early summer and autumn. The peak abundance of the conidia of aquatic hyphomycetes in an Australian upland stream occurred in late summer (Thomas, Chilvers *et al.* 1989).

While there are no fungal biomass results published for Australian lowland rivers in winter, Suter (2009) found maximum spring/summer fungal biomass on *E. pauciflora* and *E. delagetensis* leaves to be 38.6 mg/g and 104 mg/g dry mass, respectively, after 13 weeks in an Australian alpine stream. As maximum biomass on *E. camaldulensis* leaves in summer was 45-50 mg/g dry mass, Suter’s (2009) data suggest that fungal biomass on eucalypt leaves is higher under cooler conditions, but winter biomass in lowland rivers and wetlands will need to be measured directly to determine how fungal biomass varies seasonally.
6.4.3 **Fungal and Oomycete community structure**

Several authors have found that the fungal community that colonises submerged eucalypt litter is distinct from the community colonising other litter types (Bärlocher, Canhoto *et al.* 1995; Bärlocher and Graça 2002; Chauvet, Fabre *et al.* 1997; Thomas, Chilvers *et al.* 1992). My study has found that the fungal community on submerged *E. camaldulensis* leaves does not differ between rivers and wetlands at sites on three rivers. This suggests that the specialised fungal community colonising submerged eucalypt leaves is ubiquitous in regions where these species are indigenous (Thomas, Chilvers *et al.* 1992).

There have been no previous studies investigating the diversity of Oomycetes colonising submerged eucalypt litter. My study has shown that the Oomycete community shows greater variability than the broader fungal community, differing between rivers and wetlands, and between sites. As these organisms not only degrade submerged litter but also have the potential to cause disease in fish, crayfish and amphibians (Kiesecker, Blaustein *et al.* 2001; Singhal, Jeet *et al.* 1987; Söderhäll, Dick *et al.* 1991; van West 2006), these differences in community composition are potentially of ecological significance. Oomycetes are also potentially strong degraders of lipids (Beakes and Ford 1983; Mozaffar and Weete 1993), suggesting a role in attacking the oils and waxes found in eucalypt leaves.

While fungal community structure data presented here indicate the presence of fungi belonging to the Ascomycetes, Basidiomycetes, Chytridiomycetes, Zygomycetes and Oomycetes, the Oomycete community structure data represent a subset of this community (Nikolcheva and Bärlocher 2004). It was hypothesised that Oomycetes would have a stronger influence on the composition of the fungal community in standing waters than in flowing environments, and it was indeed possible to discriminate between rivers and wetlands using Oomycete community structure data, but only at the Kiewa and Murray sites. This suggests that Oomycete community structure potentially differs between rivers and wetlands but that it is influenced by site specific factors.

The structure of Oomycete communities is known to be influenced by factors such as oxygen concentration (Dix and Webster 1995), pH (Lund 1934), season (Khallil, El-Hissy *et al.* 1995; Nikolcheva and Bärlocher 2004; Roberts 1963), salinity (Padgett, Kendrick *et al.* 1988) and the composition of the substrate (Bärlocher 1992). The
requirement for oxygen and the ability to utilise specific plant components varies among taxa, with some species (e.g. *Leptomitus lacteus*) being unable to utilise sugars, instead consuming organic acids, fatty acids and amino acids. In general, Oomycetes show a preference for substrates that are rich in chitin and cellulose. These include dead insects, hemp seeds and snake skin (Park, Sorenson *et al.* 1978). This diversity and the ability of many species to tolerate harsh environmental conditions helps to explain their ability to utilise submerged plant detritus as a substrate in both early and late phases of decomposition, as substrate composition and environmental conditions change.

Changes in fungal community structure observed on submerged *E. camaldulensis* leaves are consistent with succession as the leaf substrate is consumed and its composition changes (Frankland 1982). For example, fungi adapted to consume sugars may be replaced by those able to utilise cellulose, and these fungi are then replaced by those able to utilise lignin (Kjoller and Struwe 1992), although some species of fungi may be able to utilise all of these substrates and maintain a presence throughout leaf decomposition. Moreover, fungi and stramenopiles have been found to coexist on submerged plant litter in standing water (Czeczuga, Mazalska *et al.* 2005; Czeczuga, Muszyńska *et al.* 2007; Nikolcheva and Bärlocher 2004) and maximum species diversity is seen 3-6 weeks after the leaves are immersed in a water body (Fischer, Bergfur *et al.* 2009).

### 6.4.4 Ecological implications

Results from this experiment indicate that aged *E. camaldulensis* leaves submerged in lowland rivers and floodplain wetlands during summer only represent a nutritious food source for invertebrates for about 1 month after inundation. The temporal limitations on the food value of this detritus exist because the floodplain aged leaves are low in nitrogen, and low substrate nitrogen combined with low concentrations of nitrogen in the water column compel fungal colonisers to use nitrogen conservatively, but nitrogen will be lost from the leaf when fungi reproduce and release propagules. As *E. camaldulensis* loses most of its leaves in summer (Glazebrook and Robertson 1999), the continual supply of leaves would ensure that nitrogen enriched leaves do not become scarce, but better food quality where ambient nitrogen concentrations are higher will profoundly influence the growth and reproduction of shredder communities (Gulis, Ferreira *et al.* 2006). Stable isotope studies of food webs in wetland systems (cf Bunn and Boon 1993; Hladyz, Gessner *et al.* 2009; Reid, Quinn *et al.* 2008) would help to resolve these issues.
The amount of fungal biomass accumulating on submerged *E. camaldulensis* leaves in summer is consistent with previous studies (Suberkropp 1997; Suter 2009). It can therefore be assumed that similar levels of fungal biomass are a normal component of food webs in these systems and the composition and abundance of other aquatic taxa have adapted under these conditions. However, it is likely that *in situ* fungal biomass values will be lower on leaves in rivers and wetlands as it will be exposed to consumption by invertebrates such as snails (Newell and Bärlocher 1993) and caddis fly larvae (Bärlocher and Kendrick 1975; Chung and Suberkropp 2009). Grazing by invertebrates is also likely to alter the composition of the fungal community (Sabetta, Costantini *et al.* 2000), although, there are no published studies showing that invertebrates are actually consuming conditioned eucalypt leaves in Australian floodplain wetlands. Given the C:N of these leaves, wetland invertebrates may consume other types of detritus preferentially.

While similarities can be expected between floodplain wetland environments by virtue of the absence of flow, and similar riparian vegetation, floodplain wetlands can vary widely in terms of their water quality (Goonan, Beer *et al.* 1992; Hall, Baldwin *et al.* 2006). Summer draw down may exaggerate these differences by concentrating dissolved substances in the water column, and this will strongly influence the composition of aquatic fungal communities. Water quality issues currently facing Australian floodplain wetland ecosystems include anoxic “black water” events (Howitt, Baldwin *et al.* 2007), increased salinity (Nielsen, Brock *et al.* 2003) and decreasing pH (Baldwin, Hall *et al.* 2007). While salinity, for example, has been found to reduce fungal biomass in standing water (Czeczuga, Muszyńska *et al.* 2007), it is important to determine how fungal biomass and community composition in Australian systems will be impacted by these disturbances and how this might affect the decomposition of plant detritus and detritivore food chains.

Oomycetes are a little studied group of aquatic organisms (Newell 1992), that are important elements of the decomposer community in lowland rivers and floodplain wetlands. Their presence equalises total mycelial biomass between rivers and wetlands and they may be instrumental in enriching leaf detritus with nitrogen. They are also candidates for the $^{13}$C-depleted primary producer that Bunn and Boon (1993) postulated
formed the basis of floodplain wetland food chains. However, many Oomycete species are inhibited by saline conditions (Lord and Roberts 1985; Padgett, Kendrick et al. 1988). It would therefore be of interest to investigate the decomposer community on submerged detritus in saline wetlands and determine whether this detritus becomes nitrogen enriched.

6.4.5 Method considerations

In this study, mesh bags were used to exclude invertebrate shredders from the leaf packs, so that fungal accumulation in the absence of grazing could be assessed. It should be noted that exclusion of invertebrates results in slower breakdown rates, and litter bags may also alter the micro-environment of the leaf detritus. In particular, fungal community structure will be influenced if propagules are prevented from landing on the leaf surface. Here, it is possible that accumulation of biofilm on litter bags may even have prevented new species from colonising the submerged leaves in the later phases of decomposition. This will have influenced both biomass and community structure results. Future work should compare fungal dynamics between leaf packs fixed together with buttoneers (Boulton and Boon 1991) with results from large and small mesh litter bags to determine if these effects are significant.

Loss-on-ignition has been used frequently to estimate the carbon content of soils (Hutson 1985; Wang and Bettany 1995) with the use of conversion factors specific to soil type (Bent Tolstrup and Per Åkesson 1982; Konen, Jacobs et al. 2002). This method has been used less commonly for determining leaf carbon content as more accurate methods are available, but loss-on-ignition is a cheap and rapid method to estimate carbon content given suitable conversion factors. A 50% conversion factor has been used here and elsewhere (Lindroth, Kopper et al. 2001; Robertson, Bunn et al. 1999), and is in line with Wood’s (1974) finding that the carbon content of decomposing E. delegatensis leaves was 49.9% prior to incubation, and remained stable at 47.4-52.1% after 12 months of incubation in a forest litter layer. For the purposes of this study, this method provides a valid estimate of carbon content.

The biovolume method used to determine the mycelial biomass of fungi and Oomycetes is time consuming, and may underestimate fungal biomass due to difficulties in separating it from the substrate (Frankland, Lindley et al. 1978; Newell 1992). In
addition, the procedure used to clear leaf tissue may have removed hyphae attached to the leaf surface. However, this method has been used to estimate fungal biomass by several authors (Findlay, Howe et al. 1990; Findlay and Arsuffi 1989; Newell and Hicks 1982; Newell and Statzell-Tallman 1982) and provides an opportunity to simultaneously identify the fungi present. While it has shortcomings, a more satisfactory method for determining the biomass of Oomycetes is not currently available.

Aquatic Oomycetes were traditionally sampled from aquatic environments using baiting methods (Nechwatal, Wielgoss et al. 2008; Park, Sorenson et al. 1978; Pettitt, Wakeham et al. 2002), but these methods measure the number and species of propagules, not mycelial biomass. More recently, quantitative PCR has been used to determine the abundance of Phytophthora capsici infecting plant material (Silvar, Diaz et al. 2005) as well as species belonging to Eumycota (Landeweert, Veenman et al. 2003), but in their current form these techniques are species specific. It may be possible to develop these techniques by using less specific primer pairs to quantify the biomass of the Oomycete community in environmental samples.

Both methods used to measure fungal biomass depend on standard conversion factors, and while it is valid to compare values within the data sets, caution should be used when comparing biomass between data sets that have used different methods. For example, a high abundance of yeasts in a fungal community can lead to an overestimation of fungal biomass by ergosterol concentration because yeasts have an ergosterol content (37-42 µg.mg⁻¹ (Pasanen, Yli-Pietila et al. 1999)) significantly higher than that of aquatic hyphomycetes (2.3-11.5 µg.mg⁻¹), on which the ergosterol to fungal biomass conversion factor used is based (Gessner and Chauvet 1993). As yeasts are commonly found associated with plant detritus in lakes (Lefevre, Bardot et al. 2007), it is likely that the ergosterol method overestimates fungal biomass in lentic systems. However, the ergosterol content of mycelial tissue can be substantially lower under reduced oxygen conditions (reduced from 85% to 37% of total sterols)(Nout, Bonants-Van Laarhoven et al. 1987; Safe 1973), as the biosynthesis of ergosterol requires molecular oxygen, thus the tendency toward lower oxygen concentration in standing water would place downward pressure on ergosterol concentrations. Notwithstanding error due to conversion factors, fungal colonisation of leaves is patchy, and biomass estimates can be
highly variable within a single leaf and between leaves (Chamier, Dixon et al. 1984). This spatial variability may exceed errors due to inaccurate conversion factors.

6.5 Conclusions

In this chapter I have shown that fungal and Oomycete colonisation of submerged *E. camaldulensis* leaves results in a temporary improvement in food value. This improvement coincides with periods of high Oomycete biomass in the Ovens and Kiewa systems, and higher biomass of the Eumycota in the Murray system. These spatial differences correlate with low and high flow conditions and ambient nitrogen concentrations, respectively, while temporal differences correlate with changes in the fungal community structure. Fungal biomass differed between rivers and wetlands, and results suggested that Oomycete biomass was significant during the early phase of aquatic decomposition, and after 2 months in drying wetlands and during low-flow conditions in a stream.

Exploration of aquatic fungal ecology in Australian systems is a recent endeavour and there are many questions still to be answered. For example, the relative contribution of leaching and fungal production to carbon loss from eucalypt leaves is unknown, as are the organisms mediating nitrogen enrichment in submerged leaves. While influence of ecophysiological parameters such as season and water quality is known for European and North American systems, their effects have not been measured in Australian systems. It will be particularly important to determine how resilient fungal communities are to disturbances such as salinity, black water and acidification. In wetlands, it is important to determine whether detritivores actually consume conditioned *E. camaldulensis* leaves, given the rarity of shredders in floodplain wetlands and the low nutritional value of these leaves. Leaves may simply become entrained within wetland sediments (Janssen and Walker 1999) where they are degraded by anaerobic bacteria (Boon 2006). Further refinement of methods is also needed, particularly with respect to the use of litter bags and the development of a molecular technique to quantify Oomycete biomass in submerged plant detritus.

Relative increases in the nitrogen content of decomposing leaves have been assumed to be due to increasing protein as fungal biomass accumulates. However, it is possible that leaf nitrogen is being transformed into non-bioavailable refractory organic compounds as
a result of enzymatic reactions while bioavailable carbon is lost. This would also result in reducing C:N but would reduce the food value of the leaf resource in practical terms. If this is the case, there are potential implications for detritivore food chains and carbon dynamics in lowland rivers and floodplain wetlands. In the following chapter, changes in the chemical composition of submerged *E. camaldulensis* leaves are tracked through the aquatic decomposition process in a lowland river and an associated wetland using spatially explicit analyses. Such spatially precise data allow chemical changes to be correlated with zones of enzyme activity around fungal tissues and fungal succession on submerged leaves. In particular, the distribution of organic nitrogen within decomposing leaves is mapped over time.
**Synchrotron FTIR micro-spectroscopy reveals chemical changes accompanying aquatic leaf decay.**

### 7.1 Introduction

In chapter 4, fungal dynamics on submerged *Eucalyptus camaldulensis* leaves were explored using enzyme activity to indicate the breakdown of leaf components. These data suggested that the rate of breakdown of different structural carbohydrates in these leaves differs between aquatic and terrestrial conditions and with fungal community structure. The fungal community present on these leaves changes over time and differs between rivers and wetlands (Chapter 6). As these differences will influence rates of carbon mineralisation and storage within a wetland ecosystem, it is important to determine how the chemical composition of *E. camaldulensis* leaves changes after colonisation by aquatic fungi. By knowing how fungi alter their substrate we can infer the functional significance of saprotrophic fungi colonising submerged plant material in rivers and wetlands.

In Australia’s Murray-Darling Basin, *Eucalyptus camaldulensis* is the most common tree species in riparian zones and on floodplains, and its leaf litter is an important and abundant carbon resource for aquatic ecosystems. The leaves are exposed to terrestrial decomposition processes on the floodplain before they are inundated or washed into aquatic systems. During this terrestrial phase, water soluble leaf components, including soluble polyphenols (Serrano 1992), are lost through leaching and refractory leaf components may be altered by microbial enzymes, UV light and mechanical processes. At inundation, 70-80% of the remaining soluble organic substances, including free sugars and starches, are lost within a few days (Baldwin 1999; Campbell and Fuchshuber 1995; Glazebrook and Robertson 1999; Kaushik and Hynes 1971; Suberkropp, Godshalk *et al.* 1976), so it is the more refractory structural carbohydrates derived from plant cell walls that provide energy to aquatic fungi (Chamier 1985). Plant cell walls are comprised mainly of cellulose, hemicellulose, pectin and lignin, and these polymers are extensively cross-linked.

Aquatic fungi, such as aquatic and aero-aquatic hyphomycetes, are known to produce enzymes which breakdown cellulose (Abdullah and Taj-Aldeen 1989), hemicellulose
(Chamier, Dixon et al. 1984; Suberkropp and Klug 1980), starch (Abdullah and Taj-Aldeen 1989), pectin (Chamier and Dixon 1982a; Chamier and Dixon 1982b), lipids (Abdullah and Taj-Aldeen 1989) and protein (Abdullah and Taj-Aldeen 1989). Lignin, however, is attacked by oxidative enzymes, and it has been suggested that fungi in low oxygen aquatic environments such as wetlands are not capable of degrading it, and that lignin degradation is primarily a terrestrial process (Cooke and Whipps 1993). However, fungi from both of these groups, when grown on lignin agar, have demonstrated some lignin degradation capabilities (Abdullah and Taj-Aldeen 1989; Bergbauer, Moran et al. 1992; Fisher, Davey et al. 1983). Oxidative degradation of aquatic humic substances by fungal enzymes has also been demonstrated (Claus and Filip 1998; Filip and Claus 2002).

Increases in both relative and absolute lignin content with decomposition have been noted in studies of terrestrial (Gallardo and Merino 1992) and aquatic (Suberkropp, Godshalk et al. 1976) leaf decay. It is not possible for new lignin to be made in dead leaves, but compounds which behave chemically similar to lignin may be formed. Lignin break down products that react spontaneously with each other and with carbohydrates in their immediate environment are the most likely source of this “new” lignin (Melillo, Naiman et al. 1984).

As submerged leaves are decomposed, there is often an increase in their absolute nitrogen content (Kaushik and Hynes 1971; Pidgeon and Cairns 1981; Triska and Sedell 1976), consequent reduction in the C:N ratio (Moran, Benner et al. 1989) and increase in energy content (Liakos 1986). It has been proposed that this increase in nitrogen is due to the accumulation of microbial protein (Bärlocher, Mackay et al. 1978; de la Cruz and Gabriel 1974). This was supported by the finding that fungi are able to absorb nitrogen from the water column and assimilate it into their biomass (Suberkropp and Chauvet 1995), and increases in ambient nitrate concentrations lead to accelerated leaf break down (Liakos 1986). However, studies have shown that microbial protein comprises less than 30% of the total leaf nitrogen (Odum, Kirk et al. 1979), and much of the remaining nitrogen content is not in the form of protein (Rice 1982).

Several mechanisms have been proposed to explain the absolute increases in nitrogen and lignin content observed in decaying leaves. Suberkropp et al. (1976) suggested that leaf
polyphenols complex with protein during the early stages of aquatic decay, and that these complexes are isolated in the lignin fraction. This was later supported by findings that increases in the lignin content were correlated with losses of tannins (Gallardo and Merino 1992). Odum et al. (1979) demonstrated that nitrogen enrichment is only in part due to the presence of microbial protein. For example, nitrogen contained in fungal biomass comprised only 16% of total immobilised N on submerged wood (Melillo, Naiman et al. 1984). The non-proteinaceous nitrogen fraction (~70%) is likely to be comprised of amino sugars (such as chitin and glucosamine), nitrogen containing humic acids and nitrogen rich complexes of phenol-protein, lignin-protein, chitin-protein and amino-clay minerals (Melillo, Naiman et al. 1984). Up to 50% of non-proteinaceous nitrogen in leaf detritus may be derived from chitin (Odum, Kirk et al. 1979).

Rice (1982) explained increases in nitrogen and lignin in terms of the humification of organic matter. He proposed that, following leaching, leaf material is colonised by microorganisms, and the activity of their enzymes produces reactive carbohydrates and phenols. These molecules condense with peptides and amino acids to produce nitrogenous geo-polymers. Microbes assimilate nitrogen from the water column into their biomass and exudates, and these compounds later decompose and condense with reactive carbohydrates and phenols. As humification proceeds, nitrogen is gradually transformed from relatively labile amino-sugar, amino-phenol and polypeptide forms into chemically recalcitrant heterocyclic, aromatic forms typical of humic material. Melillo et al. (1984) showed that nitrogen accumulation is proportional to the concentration of water soluble polyphenols, and that nitrogen immobilisation often corresponds to increases in absolute lignin content. They proposed a cycle of enzymatic de-polymerisation and recondensation that continues until de-polymerisation becomes inefficient in terms of energy yields (Melillo, Naiman et al. 1984).

Bärlocher et al. (1989) combined leaf leachate with fungal exo-enzymes, and demonstrated that insoluble polyphenol/protein complexes are formed. They further showed that invertebrates having alkaline gut pH or surfactants in the gut fluids were able to extract amino acids from these complexes. Gallardo and Merino (1992) suggested that nitrogen from microbial biomass could be precipitated by leaf tannins in the forest litter layer, and that this process represented the first step in the formation of humic substances.
The changes in leaf components found in previous research have been observed at the “whole leaf” scale. That is, the leaf is ground and homogenised and the chemical constituents of the homogenate are determined. However, fungal activities take place at a microscopic scale, and different components of the leaf’s anatomy may be used preferentially by decomposer organisms. So to understand the chemical changes that take places as a result of fungal activity, it is necessary to examine the chemical changes at a microscopic scale, and in a spatially explicit context.

Fourier transform infra-red spectroscopy allows us to infer the chemical composition of an organic sample by measuring the infra-red absorbance resulting from the vibration of chemical bonds. With the addition of a microscope, very small amounts of organic material can be characterised. When infra-red detectors are arranged in an array (FPA–FTIR), or where the detector scans sequential positions across the surface of the sample (FTIR mapping), a spatially explicit image of the chemical components of the sample can be compiled. The use of a synchrotron light source (S-FTIR) improves spatial and spectral resolution so that we can determine chemical changes at the micron scale, a scale similar to that of fungal tissues. While this technique has been used to chemically characterise Eucalyptus leaves (Heraud, Caine et al. 2007), and fungal tissues (Jilkine, Gough et al. 2008; Kaminskyj, Jilkine et al. 2008; Szeghalmi, Kaminskyj et al. 2007) it has never been used to investigate decomposition processes.

In this chapter, I investigate the use of infra-red spectro-microscopic imaging as a means of examining the distribution of proteins, polysaccharides, lipids and lignin in the decomposing leaves of Eucalyptus camaldulensis. Changes in distribution are tracked from a fresh leaf, to one that has been decomposing on the floodplain, then through several stages of aquatic decomposition. I was also able to show differences in the protein structures present in fresh and decomposing leaves. Given that fungal community structure and biomass are likely to differ between wetland and stream environments (see chapter 6), I compared chemical changes in leaves decomposing in these contrasting environments. The higher spatial resolution of the FTIR spectro-microscopy using a Synchrotron light source was then used to examine the distribution of these compounds within the immediate vicinity of fungal hyphae.
7.2 Materials and Methods

7.2.1 Plant material

Fresh leaves were collected from a mature *E. camaldulensis* tree on the bank of the Kiewa River at Killara, Victoria, Australia (-36.140093S, 146.955185E, 152 m elevation). Leaf discs of 9mm diameter were cut from the leaves and immediately preserved in 4% formaldehyde.

For the abiotic leaching treatment, several fresh leaves were placed a sealed 500ml polycarbonate jar with autoclaved water taken from the Kiewa River. In the biotic leaching treatment, leaves were placed in a jar with untreated water from the river. These samples were stored in the laboratory at room temperature (approximately 20°C) for 12 weeks. Leaf sections were then stored in 4% formaldehyde.

Aged leaves were collected from the leaf litter on the Ovens River floodplain (-36.162422S, 146.237914E, 137m elevation), and were dried at 25°C for a period of 4 weeks in a dehydration oven. A selection of floodplain aged leaves were then stored in formaldehyde to await histological processing, while the remaining aged leaves were transferred into litter bags made from 500 μm heavy duty nylon mesh (to limit attack by aquatic fauna) (Figure 7.1,a). Leaves were submerged at a depth of 20-30 cm at three replicate positions in the Kiewa River and in an adjacent wetland at Killara (Figure 7.1, b), and then retrieved at time intervals of 2, 15, 30, 60 and 107 days. Litter bags were stored on ice after sampling, and transported to the lab where 9 mm leaf discs were cut from partially decomposed leaves and stored in 4% formaldehyde at 4°C while awaiting histological processing.

7.2.2 Histological method

Leaf discs were dehydrated in a series of ethanol baths (70%, 90% and three lots of 100%) for 12 hours in each bath. This was followed by clearing in vegetable oil xylene for 12 hours, which was repeated three times. After this, leaf sections were soaked in paraffin wax at 58°C for 12 hours, embedded in a paraffin blocks and set prior to sectioning on a rotary microtome (Boundy, undated; Heraud, Caine et al. 2007). Transverse sections of leaf specimens, 4μm thick were mounted onto tin-oxide coated, glass slides with silver layers to provide reflectance (MirrIR low e slides: Kevley Technologies, Chesterland, OH, USA). Transverse sections of 8μm thickness were
mounted onto calcium fluoride windows (Crystran Ltd., Poole, UK) that had been coated with poly-D/L-lysine. Paraffin was removed from the sections by immersion in three xylene baths for 2 minutes each, immediately prior to infrared analysis.
Figure 7.1: Floodplain aged leaves were inserted into mesh litter bags (a), then submerged in the river and floodplain wetland (b). The wetland was an ox-bow lake on the floodplain of the Kiewa River, in north-eastern Victoria, Australia, with vegetation dominated by *Eucalyptus camaldulensis* (c).
Additional adjacent sections were prepared for examination under a compound microscope. These sections were stained with periodic acid / Schiff reagent (PAS), which binds to glycogen and carbohydrates, and crystal violet, which penetrates cell membranes and stains DNA and cytoplasm purple. Images of these sections indicating the organisation of leaf tissues and the absence of fungal hyphae in a fresh leaf, and the presence of fungal hyphae in a partially decomposed leaf are shown in Figure 7.2.

### 7.2.3 Infrared spectroscopy

Broad scale infrared images of leaf midvein and adjacent mesophyll (FPA-FTIR) were captured in both transfectance and transmittance modes using the 15× objective of an Hyperion 3000 infrared microscope (Bruker Optik GmbH, Ettlingen, Germany), with a total path length of 8 μm for both methods. The infrared microscope was equipped with a 64 × 64 element focal plane array (FPA) detector coupled to a Vertex 70 FT-IR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) operating in rapid scan mode, and located at the Australian Synchrotron (Clayton, Victoria, Australia). Opus 6.5 software (Bruker Optik GmbH, Ettlingen, Germany) was used both to control the instrument and to process the spectra. Most spectra were acquired in the “transfectance” mode whereby the IR beam passes through the sample and reflects off the supporting slide and trough the sample again to the detector. The remaining spectra were acquired in “transmittance” mode, whereby the IR beam passes through the sample and the supporting calcium fluoride window to the detector above. Total path length in both modes was 8 μm. 2×2 pixel binning was used for image frame collection, and apodization was performed using Blackman-Harris 3-term function. Data were obtained with 64 co-added scans at 4 cm⁻¹ spectral resolution. Each data set consisted of 16384 data points, collected over 16 blocks in 4×4 and 8×2 array comprising ~0.46 mm² of leaf area. For images including the leaf mesophyll, two 8×2 images were collected and are presented with the common edges abutting. Reference (background) spectra with 64 co-added scans were measured before each sample image frame.

Higher spatial resolution FT-IR images of leaf sections were acquired using an infrared microscope with a synchrotron light source (S-FTIR). Absorption spectra maps were acquired in transfectance mode using the 36× objective of an Hyperion 2000 infrared microscope (Bruker Optics GmbH, Ettlingen, Germany) with a jacketed stage purged with dry air to maintain an environment of stable humidity. A motorised stage enabled
point by point raster scanning of the sample. The microscope was coupled to a Vertex 80V infra-red spectrometer and was equipped with a narrow-band liquid nitrogen cooled MCT detector (Bruker Optik GmbH, Ettlingen, Germany) and connected to the infrared beam line 2BM1 at the Australian Synchrotron (Clayton, Victoria, Australia). This system was also controlled using Bruker’s Opus 6.5 software. The microscope has a glass knife-edge aperture which was used to define a sample area of 5×5 μm. Images used in this report had an area of less than 300×80μm (See figure captions for exact dimensions).

![Figure 7.2](image)

Figure 7.2: 8 μm cross sections of the mid-vein of *Eucalyptus camaldulensis* leaves that have been stained with periodic acid/Schiff’s (PAS). The left panels show a fresh leaf (a), and the right panels show a leaf that has been aged on the floodplain then submerged in a wetland for 2 days (b). Images are shown at 100x (i) and 400x (ii) magnification. While no fungal tissue can be observed in the fresh leaf, in the partially decomposed leaf, darkly staining fungal hyphae have replaced the phloem tissues and much of the mesophyll. Bundle sheath cells appear to have collapsed in the partially decomposed leaf. Fungal hyphae can be seen on either side of the fibre sheath and within the mesophyll of the partially decomposed leaf (b(ii)). X = xylem; P = phloem; FS = fibre sheath; E = epidermis; BS = bundle sheath; F = fungal tissue; M = mesophyll; OV = oil vesicle.

In Figure 7.7, (a) and (b), the specimen was mapped in 5 μm steps, whereas 2 μm steps were used in Figure 7.7, (c). Data were collected with 64 co-added scans, spectral
resolution of 4 cm$^{-1}$, and with Happ-Genzel apodization function. Reference spectra were collected after each 8 sample data points with 128 co-added scans.

For both FPA-FTIR and S-FTIR data were collected in the spectral region of ~3800 to 750 cm$^{-1}$.

7.2.4 Band assignments

The amide bands (amide I (1700-1637 cm$^{-1}$), amide II (1543-1527 cm$^{-1}$) and Amide III (1330-1290 cm$^{-1}$)) are generally considered to be indicative of the presence of proteins. Absorbance in the amide I band indicates vibration of carbonyl (C=O) bonds from peptide bonds in proteins, but may also include absorbance due to carbonyl groups in esters and aromatic compounds such as quinones.

Cellulose and hemicellulose consists of pyranose monomers linked by glycosidic (>C-O-C<) bonds (Kacuráková, Capek et al. 2000). They are found in the middle lamella, primary and secondary cell walls (Esau 1977; Raven, Evert et al. 1992). In infrared spectra, they absorb strongly in the region associated with (C=O-C) and (C-OH) bonds (1180-900 cm$^{-1}$). This frequency range will be referred to as the polysaccharide band.

Lignin is a complex three dimensional polymer comprised mainly of phenylpropanoid units, with linkages and cross-linkages via carbon-carbon (C-C), ester (C-O-C) and a variety of other linkages (Hammel, 1997). Lignin is found in the middle lamella, and in the primary and secondary cell walls of xylem and fibres. As it is highly aromatic, it is associated with the vibration of the C-C bonds of phenyl rings (1503 cm$^{-1}$), and ether linkages associated with aromatic rings (1231 cm$^{-1}$). The band at 1503 cm$^{-1}$ will be referred to as the aromatic band, while the band at 1231 cm$^{-1}$ will be referred to as the ether linkage band. Together, these frequencies are referred to as lignin bands.

Plant oils and waxes are comprised of long-chain aliphatic “fatty” acids. Oils contain triglycerols, which are in turn comprised of three fatty acids linked via ester bonds to glycerol. Waxes are comprised of long-chain fatty acids linked to a long-chain alcohol via an ester bond. Together with sterol and isoprenoid compounds, these compounds are known as lipids. The carobonyl band at 1740-1730 cm$^{-1}$ is associated with ester
functional groups ((C=O)-O-C) from fatty acids, esters and lignin. It has been used here to indicate the presence of lipids.

The spectral bands used in the analysis of *Eucalyptus camaldulensis* leaf tissue and microbial signals are primarily based on the assignments used by Heraud *et al.* (Heraud, Caine *et al.* 2007) in their analysis of *Eucalyptus botryoides* leaves. Assignment of additional bands is based on Silverstien *et al.* (1974), Hori and Sugiyama (2003), Robert *et al.*, (2005), Seoudi *et al.*, (2005) and Bandekar and Krimm (1979) (see Table 7.1).

Table 7.1: Bands of interest in the FT-IR spectra obtained from decomposing leaf material in the range 1800-900 cm\(^{-1}\). The column labelled “Wave Number” indicates the frequency limits between which each band was integrated, “Band Assignment” indicates the molecular vibration responsible for absorbance in the stated frequency range (\(\nu = \) stretch; \(\nu_s = \) symmetric stretch; \(\nu_{as} = \) asymmetric stretch \(\delta = \) deformation (bend)). The reference from which this assignment was sourced is listed in the third column, and an indication of the probable source of these vibrations within leaf material is indicated in the “notes” column.

<table>
<thead>
<tr>
<th>Wave Number (cm(^{-1}))</th>
<th>Band Assignment</th>
<th>References</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1740-1730</td>
<td>(\nu(C=O))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>Ester functional groups from fatty acids, esters and lignin</td>
</tr>
<tr>
<td>1729-1713</td>
<td>(\nu(C=O))</td>
<td>Silverstein <em>et al.</em>, 1974</td>
<td>Aliphatic aldehydes and ketones</td>
</tr>
<tr>
<td><strong>Amide I band</strong></td>
<td>(\nu(C=O), \nu_s(C=C))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>Mainly from proteins, also olefinic and aromatic double bonds</td>
</tr>
<tr>
<td>1700-1637</td>
<td>(\nu(C=O)) conjugated to (C=C)</td>
<td>Silverstein <em>et al.</em>, 1974</td>
<td>From quinones, aromatic aldehydes and aromatic ketones</td>
</tr>
<tr>
<td>1690-1655</td>
<td>(\nu(C=O)) and (\nu(C=O))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>From pectins and lignins, respectively.</td>
</tr>
<tr>
<td><strong>Amide II band</strong></td>
<td>(\nu(C=H)) and (\delta(N-H))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>From proteins</td>
</tr>
<tr>
<td>1543-1527</td>
<td>(\nu(C=C))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>Aromatic skeletal vibration from lignins</td>
</tr>
<tr>
<td><strong>Aromatic Band</strong></td>
<td>(\nu(C=C))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>From pectins</td>
</tr>
<tr>
<td>1441-1396</td>
<td>(\nu_s(C=O))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>From pectins</td>
</tr>
<tr>
<td>1389-1259</td>
<td>(\nu_s(NO_2))</td>
<td>Silverstein <em>et al.</em>, 1974</td>
<td>Nitrates, nitramines, conjugated nitro compounds, aromatic nitrates</td>
</tr>
<tr>
<td>1342-1266</td>
<td>(\nu(C-N))</td>
<td>Silverstein <em>et al.</em>, 1974</td>
<td>Aromatic amines</td>
</tr>
<tr>
<td><strong>Amide III band</strong></td>
<td>(\nu(C-H)) and (\delta(N-H))</td>
<td>Bandekar and Krimm, 1979</td>
<td>From proteins</td>
</tr>
<tr>
<td>1330-1290</td>
<td>(\nu_s(NO_2))</td>
<td>Silverstein <em>et al.</em>, 1974</td>
<td>Organic nitrates</td>
</tr>
<tr>
<td><strong>Ether Linkage</strong></td>
<td>(\nu(C-C), \nu(C-O))</td>
<td>Heraud <em>et al.</em>, 2007</td>
<td>From lignins</td>
</tr>
<tr>
<td>1284-1185</td>
<td>(\delta(N-H))</td>
<td>Seoudi <em>et al.</em>, 2005</td>
<td>From N-acetyl side chain in chitin</td>
</tr>
<tr>
<td>1260-1180</td>
<td>(\nu_s(C-O-H))</td>
<td>Silverstein <em>et al.</em>, 1974</td>
<td>From polysaccharides, influenced by hydrogen bonding</td>
</tr>
<tr>
<td>1250-1020</td>
<td>(\nu(C-N))</td>
<td>Silverstein <em>et al.</em>, 1974</td>
<td>Unconjugated C-N in aliphatic amines</td>
</tr>
</tbody>
</table>
Polysaccharides | $\nu$(C-O-C) | Heraud et al., 2007 | From polysaccharides
--- | --- | --- | ---
1180-900 | $\nu$(C-O-C) | Hori and Sugiyama, 2003 | From $\beta$-1,4-glycosidic bonds from microcrystalline cellulose
1180-1140 | $\nu$(C-O-C) | Silverstein et al., 1974 | Secondary alcohols from sugars and phenols
1085-1050 | $\nu$(C-O-H) | | $\beta$-1,4-glucans from microcrystalline cellulose
1033 | $\delta$(C-O-C) | Hori and Sugiyama, 2003; Robert et al, 2005 | 

7.2.5 Data processing and image analysis

Spectral maps were constructed using Opus 6.5 software (Bruker Optik GmbH, Ettlingen, Germany). The Opus default integration method was used, whereby the peak integration area is defined as the area under the curve between two selected wave numbers, with a base line taken between the same two points. The wave number limits for each band are shown in Table 7.1. Chemical maps were then constructed where x and y co-ordinates are the spatial co-ordinates of a given sample, and the z co-ordinate is the relative absorbance. Z-values were represented by a colour scale (Opus “rainbow” colour scale) where lightest pink indicates the maximum and darkest blue the minimum relative absorbance. Minimum absorbance was forced to zero excluding negative values and maximum absorbance was set to a consistent value for maps of all samples at a given frequency. High absorbance in a given band was correlated with structures and tissue types within each leaf by overlaying IR absorbance contours on a visual image of each sample. Images presented as absorbance contours overlaid on the bright field images are used to highlight these correlations with plant structures, but are essentially the same as full colour maps.

Spectra to be used in multivariate analysis were extracted from IR chemical images using Opus 6.5 software. Firstly, maps indicating the distribution of absorbance in the amide I band were created, and then spectra with a high absorbance were extracted from points in the map.

Chemometric analysis of this data was carried out using “The Unscrambler” software (CAMO Software AS, Oslo, Norway). Individual spectra with high absorbance in the amide I band were sampled from Opus maps then imported into “The Unscrambler”. These spectra were normalized by peak area, whereby the area under the curve was
equalised for all spectra. The second derivative of the Amide I peak (1660-1630 cm⁻¹) was then calculated using the Savitzky Golay function and 7 point smoothing. A statistical examination of the derivatised data was carried out using Principal component analysis (PCA), in order to determine whether differences in absorbance patterns in the amide I band were significantly different between fresh *E. camaldulensis* leaves and those that had undergone aquatic decomposition for 2 days. PCA employed leverage correction, and the principle components were recalculated after removing outliers.

### 7.2.6 Light microscopy

Two sets of leaf sections, taken immediately adjacent to those used in FT-IR spectro-microscopy, were stained using PAS and Crystal violet, respectively. These sections were examined at 40×, 100× and 400× magnification using an Olympus BX 51 compound microscope equipped with an Olympus DP12 digital camera (Olympus Australia, Pty. Ltd., Oakleigh, Victoria, Australia). Images were processed using Olysi software (Soft Imaging System, Münster, Germany).

### 7.3 Results

Anatomical features of the vascular tissue in *E. camaldulensis* leaves are shown in Figure 7.3. Vascular traces are comprised of phloem fibres, phloem and xylem, and encased in a bundle sheath. Cells comprising xylem and the fibre sheath cells are lignified, while other vascular and non-vascular cells are not. In the mid-vein, vascular traces have the distinctive appearance of a “smilie face”. Epidermal cells are impregnated with waxes and oils known to inhibit fungal colonisation (Canhoto and Graça 1999). Anatomical structures have been identified with reference to Esau (1977), Keating (1984), James and Bell (1995), Ali *et al.* (2009) and Burley *et al.* (1971). Observations regarding the spatial distribution of infra-red absorbance in various bands will refer to leaf structures as identified here.

Very few fibre sheath cells are observed in the fresh leaf sample (Figure 7.3 (b)), as a young leaf was selected to minimise the chance of fungal infection. This differs from more mature leaves, such as the floodplain aged and partially decomposed leaves used in this experiment. This is because the structure of the eucalyptus leaf vascular traces varies with leaf age (England 2001). Young leaves may have very few fibres around the vascular bundle, while in mature leaves a fibre sheath encircles the mid-vein close to the
petiole (Figure 7.3, (a)), and fewer fibres are observed as the diameter of the leaf trace decreases.

7.3.1 The chemical and structural components of fresh leaves

No fungal organisms were observed in sections of fresh leaf (Figure 7.4, a) or in fresh leaf sections from the abiotic and biotic leaching treatments (Figure 7.4, b & c). Phloem tissues were present in the fresh leaf between the xylem and the bundle sheath, but there were very few fibre sheath cells. With abiotic leaching (Figure 7.4, b) few structural changes were evident, but where leaching was accompanied by the activities of non-fungal microorganisms from the water column (biotic) (Figure 7.4, c) the integrity of the leaf began to break down. In this case, fissures began to develop on either side of the fibre sheath and between the vascular bundle and the epidermis. There was also some disintegration of the palisade mesophyll. As both leaching treatments were sealed after water was added, it is likely that lack of oxygen inhibited fungal colonisation and the breakdown observed in the biotic leaching treatment was due to anaerobic bacteria.
Figure 7.3: Anatomical Structure of the mid-vein of *Eucalyptus camaldulensis*. Above we see a line drawing (a) and an image of an 8µm cross section of the midvein of an *E. camaldulensis* leaf (b). The leaf has been stained with periodic acid/Schiff’s (PAS) and is shown at 200x magnification. X = xylem; P = phloem; FS = fibre sheath; E = epidermis and cuticle; BS = bundle sheath; PM = palisade mesophyll; SM = spongy mesophyll; C= collenchyma.

Lignin content in leaves is represented by absorbance at 1503 and 1231 cm$^{-1}$ (see Table 7.1). In fresh leaves, the spatial distribution of these signals was tightly constrained in the xylem (Figure 7.5, a (ii) & (iii)), but absorbance decreased and became more diffuse with leaching and terrestrial aging (Figure 7.5, b, c & d (ii) and (iii)). Absorbance due to the
presence of aldehydes and ketones was low in the fresh leaf sample (Figure 7.5, a (iv)), and mainly associated with the bundle sheath.

FPA-FTIR data indicate that polysaccharides were present throughout the mid-vein of fresh leaves, and in all tissue types (Figure 7.5, a,(v)). Images that also included a portion of the mesophyll (Figure 7.6) indicate that most of the polysaccharides present in the mesophyll of the fresh leaf (Figure 7.6, a) were lost during abiotic (Figure 7.6, b) or biotic (Figure 7.6, c) leaching, and the polysaccharides that remained in the floodplain aged leaf were confined to the vascular tissues (Figure 7.6, d).

The presence of proteins is indicated by absorbance in the amide bands (I, II, and III), which indicate vibrations from C=O, NH$_2$ and C-N, respectively, from within peptide bonds. Absorbance in the amide I band was at its highest in the fresh leaf (Figure 7.5, a (i)) and this absorbance was spatially correlated with the areas of the palisade mesophyll adjacent to the vascular tissue. Relative absorbance in the amide I band was also high in the phloem, and absorbance in the amide II and III bands in this area supports a conclusion that this area contained protein. Areas of high absorbance in the amide II band were also seen in the collenchyma, but this was not corroborated by amide I data and may be due to the presence of lignin absorbing at similar frequencies. S-FTIR mapping of a segment of the mid-vein showed that amide I absorbance had a patchy distribution in the fresh leaf (Figure 7.7 (a)).

The chemical and structural components of terrestrial aged leaves

The terrestrially aged leaf (Figure 7.8, a & b (i)) had been colonised by fungi, which can be seen between the xylem and fibre sheath in the vascular tissue. Phloem tissues were no longer present. Large amounts of mesophyll tissue were also absent, and this area was networked with fungal hyphae (Figure 7.8, a (i)). Reproductive structures were observed close to, or protruding through, the epidermis (Figure 7.8, a (i) & c (ii)). Hyphae were absent from oil vesicles.

In the terrestrially aged leaf section (Figure 7.5, d (i)) the area of high amide I absorbance was smaller than in the fresh leaf, and was associated with fungal tissues rather than leaf tissues. S-FTIR mapping of a segment of the mid-vein in a terrestrially aged leaf is
shown in Figure 7.7, b (i). This finer scale image shows that amide I distribution was correlated spatially with fungal tissues.

Figure 7.4: Compound microscope images leaf sections. Images are shown at 200× magnification and stained with PAS. The fresh leaf (a), the leaf that was leached in autoclaved water (b), and the leaf that was leached in non-sterile river water (c) show no sign of fungal colonisation. The leaf that was found on the floodplain (d) is extensively colonised with fungal hyphae, which is observed as darkly staining tissues surrounding the xylem and fibre sheath, and replacing the phloem and mesophyll. **Fungal hyphae (H) are networked throughout the mesophyll region of the leaf found on the floodplain.**
Figure 7.5: Broad scale images of the leaf mid-vein, acquired using the Focal Plane Array FT-IR micro-spectrometer (x15 magnification). Here we show four micrometer sections through *E. camaldulensis* leaves that were (a) removed from a living tree (fresh); (b) leached in autoclaves river water; (c) leached in untreated river water; and (d) found on the surface of the floodplain (floodplain aged). Bright field images of the unstained leaf section are shown in the leftmost column, while images showing the spatial distribution of absorbance in the following bands are shown in subsequent columns: (i) 1700-1637 cm\(^{-1}\); (ii) 1530-1486 cm\(^{-1}\); (iii) 1284-1185 cm\(^{-1}\); (iv) 1729-1713 cm\(^{-1}\); and (v) 1180-900 cm\(^{-1}\). Lightest pink indicates highest absorbance while darkest blue indicates lowest absorbance.
Figure 7.6: Loss of polysaccharides on leaching. Here, the same leaf sections that were shown in Figure 7.4 (Bright field) and Figure 7.5 are viewed with portions of the leaf mesophyll. FPA-FTIR spectral images show absorbance in the polysaccharide band (1800-900 cm\(^{-1}\)) of a fresh leaf (a), a leaf that has been leached abiotically (b) and biotically (c) in the laboratory, and of a leaf found on the floodplain. Maximum absorbance is fixed at 120 au (lightest pink) and
minimum absorbance is 0 au (darkest blue). The highly absorbing are to the left of the midvein in image (d) is a minor vascular trace.
The amide III band is associated with vibrations from proteins, but the vibrations from the N-acetylated side-chains in chitin and organic nitrates may also absorb in this region (see Table 7.1). In the terrestrially aged leaf (Figure 7.9, c (ii)) there was no absorbance in this band associated with xylem, but areas of higher absorbance correlated spatially with the fibre sheath cells, where microscopic observation suggests that fungi may have been attacking the middle lamella.

Many fungal fruiting bodies were observed in both terrestrially aged and aquatically decomposed samples. The structures tended to absorb in the amide I band (Figure 7.10, a) with variable responses in the amide II and III bands (Figure 7.10, b & c). Spectra indicated that aromatics (Figure 7.10, d), lipids and polysaccharides (data not shown) were also present. Areas of higher absorbance values in the amide I band that are not spatially correlated with higher absorbance in Amide II and III bands within the fruiting body, but with aromatic bands suggest that protein-like substances are associated with aromatic structures in fungal tissues.

While in the fresh leaf absorbance in the lignin bands was tightly correlated with cell walls in the xylem, the signal became more diffuse and extended over a larger proportion of the mid-vein region in the leached (Figure 7.5, b and c (ii) and (iii)) and floodplain aged leaves (Figure 7.5, d, (ii) and (iii)).

All polysaccharides present in the terrestrially aged leaves (Figure 7.6, d) were associated with leaf vascular tissues and epidermis, and with fungal tissues. High relative absorbance in the polysaccharide band was concentrated in the vascular bundle of the terrestrially aged leaf, as it was in the fresh leaf, but the exclusive association with xylem was expanded to include phloem fibres and other cell types. FPA-FTIR and S-FTIR images indicate a strong correlation between the spatial distribution of lignin and polysaccharides and leaf vascular tissues, as might be expected given these molecules are extensively cross-linked in cell walls (Figure 7.5, d (ii), (iii) and (v)). S-FTIR images indicated that lignin and polysaccharides were present in xylem and fibre sheath cells, although fibre sheath cells had higher absorbance in the polysaccharide region and lower absorbance in the lignin regions, relative to xylem (Figure 7.7, b (ii), (iii) and (v)). Areas of higher absorbance in both aromatic and polysaccharide bands were found immediately
adjacent to fungal tissue (around the “eye” and at the base of the “smile”), with absorbance then gradually declining with increasing distance.

Discrete molecules of aromatic aldehydes, such as veratryl aldehyde (3,4-dimethoxybenzaldehyde), are formed as products of the break down of lignin by oxidative enzymes (Tien and Kirk 1984), and their presence in dead leaves indicates regions of microbial enzyme activity. In FTIR, aldehydes have strong absorbance in the carbonyl band at 1729-1713 cm$^{-1}$. FPA-FTIR and S-FTIR images showing the distribution and concentration of aldehydes in the mid vein of the terrestrially aged leaf are shown in Figure 7.5 (d, (iv)) and Figure 7.7 (b, (iv)), respectively. These images indicate that oxidative enzyme activity towards lignin was most active while the leaf was on the floodplain, and that this activity was concentrated in areas close to fungal hyphae.
Figure 7.7: S-FTIR spectral maps of sections through the leaf mid vein.

Fine scale maps of sections through the leaf mid-vein, acquired using an (S-FTIR) (x 36 magnification). Here we show chemical maps extending from the upper epidermis towards the centre of the leaf mid-vein and including tissues from the xylem. All maps are derived from the same 4µm $E.\textit{camaldulensis}$ leaf sections as FPA-FTIR images and have a pixel size of 5µm x 5µm. Here we show (a) a fresh leaf, the segment is 80 x 330µm, and uses 5µm step between pixels; (b) a floodplain aged leaf, the segment is 80 x 212µm, and uses a 5 µm step between pixels; and (c) a floodplain aged leaf that has been submerged in a floodplain wetland for 2 days, the segment is 40 x 90µm and uses a 2µm step between pixels. Bright field images of the unstained leaf section are shown in the left-most column, while images showing the spatial distribution of absorbance in the following bands are shown in subsequent columns: (i) 1700-1637 cm$^{-1}$; (ii) 1530-1486 cm$^{-1}$; (iii) 1284-1185 cm$^{-1}$; (iv) 1729-1713 cm$^{-1}$; and
(v) 1180-900 cm⁻¹. Lightest pink indicates highest absorbance while darkest blue indicates lowest absorbance.
The area of the sample shown in the infra-red images is indicated by the red matrix on the visible image.

Figure 7.8: Fungal features in wetland and river decomposed leaves.
Fungal hyphae (H) are networked throughout the mesophyll in a leaf found on the floodplain (a (i)), and in a leaf that had been decomposing in the river for 2 days (a (ii)). Fungal hyphae are shown to extend through the space formerly occupied by the phloem in a leaf found on the floodplain (b (i)), and in a leaf that had been in the river for 15 days (b (ii)). Leaf epidermis begins to break down after 60 days in the wetland (c (i)), and in the river (c (ii)). Fungal reproductive structures (R) can also be observed in a (i), b (i), and c (ii). Images are taken at 400x magnification using a compound microscope. Sections in a (i), b (i), c (i) and c (ii) are stained with PAS. Sections in a (ii) and b (ii) are stained with gentian violet.
Figure 7.9: Contour map images of absorbance in amide bands I, II and III.

Contour images are shown for a fresh leaf (60*225 µm, epidermis at base) (i), a leaf found on the floodplain (60*120 µm, epidermis at base) (ii), a leaf that decomposed in the river for 15 days (60*150 µm, epidermis at base) (iii) and a leaf that decomposed in the river for 107 days (60*120 µm, epidermis at top). Absorbance is mapped for the amide I band (a), the amide II band (b), and the amide III (c). Each section extends from the epidermis (E) through collenchyma (C) (i) or fungal hyphae (H) (ii-iv) to fibre sheath cells (FS) (absent in iv). Phloem cells (P) (i) or more fungal tissues (H) then separate the fibre sheath cells from xylem (X) tissues.
Figure 7.10: Fungal reproductive structure with contours indicating the distribution of absorbance from amide bands and aromatics. This feature was located in the section of the leaf that had been
submerged in the river for 15 days (240*220 µm). Contour maps of absorbance in amide I (a), amide II (b), amide III (c) and aromatic bands (d) are shown.
7.3.3 The chemical and structural components of leaves partially decomposed under aquatic conditions

After 2 days in the water the distribution of fungal tissue in aged leaves continued to be centred on the leaf vascular traces, with hyphae extending into the remaining mesophyll. The image seen in Figure 7.8, b (i) shows a vascular trace from the terrestrially aged leaf section cut at an oblique angle. Fungal tissues (H) are located between xylem cells and the fibre sheath, and these hyphae appear to be extending longitudinally along the trace. Figure 7.8, b (ii) shows a longitudinal aspect of a vascular trace of the leaf that had decomposed in the river for 15 days. Magenta coloured fungal hyphae (H) are seen extending within the vascular trace between the striated xylem and the purple fibre sheath cells. This pattern of colonisation suggests that fungal tissues have invaded the vascular trace and are extending along them both internally and externally. At the same time, hyphae extend into the mesophyll, breaking down the tissues and forming reproductive structures (R) (Figure 7.8, a (i), b (i) & c (ii)).

Absorbance in the amide II band was poorly resolved in FPA-FTIR spectra. However, S-FTIR spectral maps of leaves decomposing under aquatic conditions showed that absorbance in this band was widespread over the leaf sections, similar to the distribution of amide I absorbance. In the early stages of aquatic decay in the river (Figure 7.9 b (iii)) higher absorbance in the amide II band was correlated with fungal tissues. In the later stages of decay there was no differentiation between fungal and leaf tissues (Figure 7.9, b (iv)).

Absorbance in the amide III band is at times associated with fungal tissues, and at other times associated with phloem fibresa or xylem. In leaves that had decayed in the river for 15 days high absorbance in the amide III band correlated with fungal tissues (Figure 7.9, c (iii)). After 107 days in the river, areas of stronger absorbance were located within the xylem and were not associated with fungal tissues (Figure 7.9, c (iv)). Leaves that had decayed for 2-30 in the wetland showed higher amide III absorbance associated with the fibre sheath and fungal tissues (data not shown).

After 15 days under aquatic conditions in the wetland (Figure 7.11, b) leaves exhibited a loss of structural integrity, with a tendency to develop fissures on the periphery of the
xylem. Most of the non-vascular tissue, except for the epidermal cells, had disappeared. After 30 days of aquatic decomposition (Figure 7.11, c) xylem tissues tended to separate from other tissues upon sectioning and mesophyll was absent. After 60 days there was evidence of breakdown of the epidermal cell layer, and fungal attack of lignified tissues. This trend continued until 107 days when the leaf consisted of the remnants of vascular tissues and epidermal cells (Figure 7.11, e).

Leaves taken from aquatic conditions in the wetland had lower absorbance in the amide I band than fresh or terrestrially aged leaves. However, this lower absorbance was more widely distributed and extended over the entire mid-vein (Figure 7.12, a-e (i)). Sections of leaves taken from the river had lower absorbance in this band than did fresh leaves, but higher absorbance than floodplain aged leaves (Figure 7.13, a-e (i)). S-FTIR shows areas of higher absorbance in river samples correlated spatially with fungal tissues (Figure 7.9 a, (iii) & (iv)).

With decomposition, whether in the wetland or the river, the absorbance due to aromatics became more diffuse, distributed over the area of the xylem but not tightly confined to cell walls. FPA-FTIR images of aquatically decomposing leaves indicate similar patterns of absorbance due to lignin and polysaccharides to those seen in the terrestrially aged leaf (Figure 7.12 and Figure 7.13, a-e (ii), (iii) and (v)). Absorbance due to the presence of aldehydes indicates that lignin was being decayed enzymatically during aquatic decomposition (Figure 7.12, a-e, (iv) and Figure 7.13, a-e, (iv)). Temporal trends in this process differed between the river and wetland (see below).

Polysaccharides were associated with leaf vascular tissues and fungal tissues in leaves decaying under aquatic conditions (Figure 7.12, a-e, (v) and Figure 7.13, a-e, (v)). High absorbance in the polysaccharide band correlated spatially with xylem and phloem fibres in samples decomposing underwater for 2, 15 and 30 days. In later samples from the wetland the phloem fibres were absent and absorbance in this band was lower. In later samples taken from the river absorbance in this band was higher than in earlier stages of aquatic decay. Some phloem fibres were present and absorbing in this band in the 60 days river sample, and in addition to xylem, fungal tissues had high absorbance in this band in the 107 day river sample.
Carbonyl vibrations from lipids, waxes and oils are represented spectroscopically by absorbance in the ester carbonyl band at 1740-1730 cm$^{-1}$. Leaf samples were saturated with paraffin as part of the sample preparation process, but paraffin does not contain ester groups. It is therefore unlikely that absorbance in this band is due to residual paraffin. FPA-FTIR images in this wave band (not shown) revealed that lipids persisted in the leaf cuticle and epidermis for at least 60 days after entering the aquatic environment. Absorbance in this band was also observed within vascular tissues and associated with fungal material. As lignin is also known to absorb in these frequencies, absorbance associated with xylem was probably due to the presence of lignin.
Figure 7.11: Compound microscope images of sections of terrestrially aged leaves that have partially decomposed in a wetland. Images are shown at 200x magnification and stained with PAS. After 2 days in the wetland (a) fungal hyphae (H) can be observed around and within the vascular bundle...
and throughout the remaining mesophyll (M) tissue. After 15 days (b) fungal distribution is similar to day 2, but fissures are developing in the mid-vein. After 30 days (c), 60 days (d), and 107 days (e) leaf disintegration was continuing, and the fibre sheath had decayed. Xylem (X) and the epidermis (E) remained intact.
Figure 7.12: Broad scale images of the leaf mid-vein, acquired using the Focal Plane Array FT-IR micro-spectrometer (x15 magnification). Here are shown four micrometer sections through floodplain aged *E. camaldulensis* leaves that have been submerged in a floodplain wetland for (a) 2; (b) 15; (c) 30; (d) 60; and (e) 107 days. Visible light images of the unstained leaf section are shown in the left-most column, while images showing the spatial distribution of
absorbance in the following bands are shown in subsequent columns: (i) 1700-1637 cm\(^{-1}\); (ii) 1530-1486 cm\(^{-1}\); (iii) 1284-1185 cm\(^{-1}\); (iv) 1729-1713 cm\(^{-1}\); and (v) 1180-900 cm\(^{-1}\). Lightest pink indicates highest absorbance while darkest blue indicates lowest absorbance.

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<table>
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<th>1284-1185 cm(^{-1})</th>
<th>1729-1713 cm(^{-1})</th>
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<td>(\beta)-glycosidic linkages</td>
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<tr>
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<td>(ii)</td>
<td>(iii)</td>
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</tr>
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Figure 7.13: FPA-FTIR images of the leaf midvein from leaves submerged in the Kiewa River.

Shown here are four micrometer sections through floodplain aged *E. camaldulensis* leaves that were submerged in a floodplain wetland for (a) 2; (b) 15; (c) 30; (d) 60 and (f) 107 days. Visible light images of the unstained leaf...
section are shown in the left-most column, while images showing the spatial distribution of absorbance in the following bands are shown in subsequent columns: (i) 1700-1637 cm\(^{-1}\); (ii) 1530-1486 cm\(^{-1}\); (iii) 1284-1185 cm\(^{-1}\); (iv) 1729-1713 cm\(^{-1}\); and (v) 1180-900 cm\(^{-1}\). Lightest pink indicates highest absorbance while darkest blue indicates lowest absorbance.
Figure 7.14: Compound microscope images of sections of terrestrially aged leaves that have partially decomposed in a river. Images are shown at 200x magnification and stained with PAS. After 2 days in the river (a) fungal hyphae can be observed around and within the vascular bundle and throughout the mesophyll, some mesophyll tissue is still present. After 15 days (b) fungal distribution is similar to day 2. Loss of integrity in leaves is greater than in the wetland, possibly due to sand blasting effects from suspended river sediments. Sand grains in the samples cause sections to disintegrate as the microtome makes its cut. After 30 days (c), 60 days (d), and 107 days (e) leaf disintegration is continuing, and the fibre sheath has decayed. Xylem and the epidermis remain intact and fungal reproductive structures become abundant.
Samples that had been submerged in the river showed a similar degree of fungal colonisation to those submerged in the wetland, and fungal hyphae were distributed throughout similar plant tissues (Figure 7.14). However, breakdown of the fibre sheath cells was evident after 30 days in the river (Figure 7.14, c) but was not observed until after 60 days in the wetland (Figure 7.11, d). A large fungal reproductive structure can be seen on the lower side of the midvein in the leaf section from 107 days in the river (Figure 7.14, e). Differences were observed in the structure of fungal hyphae and reproductive structures between river and wetland samples, but no attempt was made to identify or quantify the species present.

The magnitude of absorbance in the aromatic band (1503 cm$^{-1}$) (Figure 7.12, a-e, (ii)) did not change appreciably over time for leaves decaying in the wetland, while leaves decaying in the river showed increasing absorbance from lignin over time (Figure 7.13, (ii)). Absorbance in the ether linkage band (1231 cm$^{-1}$), was associated with the xylem and fibre sheath in submerged leaves. In the wetland (Figure 7.12, (iii)) absorbance was high over the first 30 days, particularly at the margins of the xylem, and then declined. In the river (Figure 7.13, (iii)) absorbance was moderate at day 2, declined at day 15, and then continued to increase over the remainder of the experimental period. The areas of highest absorbance were found at the margins of the xylem, where tissues were more dense.

Absorbance due to the presence of aldehydes indicated that lignin was being decayed enzymatically during the early stages of aquatic decomposition (up to day 30) in the wetland (Figure 7.12, c-d (iv)). In the river, activity declined from day 2 to day 15 (Figure 7.13, b & c (iv)), then continued to increase reaching a maximum at 60 days (Figure 7.13, d (iv)). This was consistent with changes in absorbance due to lignin. High absorbance due to aldehydes can also be seen in the large fungal reproductive structure in the lower portion of the river image for 107 days (Figure 7.13, e (iv)). Figure 7.7, c (iv) shows absorbance due to aldehydes in a sample submerged for 2 days in the wetland.
mapped using S-FTIR. This image shows that aldehydes were distributed around the periphery of the fungal hyphae in the aquatically decomposing leaf.

7.3.5 Chemical gradients observed in decomposing leaf material using S-FTIR

The area around hyphae in the S-FTIR spectral map seen in Figure 7.7 (c) was examined more closely in order to detect chemical changes due to the activity of fungal extracellular enzymes. Eight spectra were extracted from a segment of the 2 day aquatically decomposed sample that extended from the hyphal tissue (0 µm) into the xylem (19 µm). These spectra are shown in Figure 7.15. These data indicate the concentration of polysaccharides, ether linkages, aldehydes & ketones and lipids changes over a distance of 19 µm in a way that is consistent with enzymatic break down of the leaf components.

Polysaccharide concentrations were lowest close to the hypha and increased with increasing distance from fungal hyphae. Specifically, peaks at 1161, 1060 and 1033 cm⁻¹ are indicative of cellulose (Hori and Sugiyama 2003) and the height of these peaks increased with distance from the hypha. This is pattern is consistent cellulose breakdown by extracellular enzymes that cleave β-1,4-glycosidic linkages between pyranose rings.

Lignin is composed of phenyl rings connected by ether (and other) linkages. The band at 1530-1486 cm⁻¹, indicating the presence of phenyl rings, was not well resolved in spectra correlating with fungal tissues, but resolution improved with increasing distance into xylem tissues (blue spectra). Relative absorbance in this band decreased markedly between 8 and 15 µm from the hypha, possibly indicating a transition between altered and unaltered xylem. The ether linkage band (1284-1185 cm⁻¹) showed decreasing absorbance over 10-15 µm extending away from the hypha, and increasing absorbance over the remaining distance. Moreover, there was a shift in the peak maxima to higher frequencies with increasing distance, indicating a change in the conformation of this bond type, possibly due to more extensive hydrogen bonding.

The amide I and II bands (1700-1637 and 1543-1527 cm⁻¹), indicating protein, were present over the first 1-6µm, although the peaks were poorly resolved. Over the latter 6-19 µm, these peaks were absent, and a peak at 1590 cm⁻¹ was observed. The peak at 1590 cm⁻¹ is consistent with C=C stretching is aromatic rings from lignin (Michell 1992).
The carbonyl peak at 1740-1730 cm\(^{-1}\) was absent over the first 4-9 µm, corresponding spatially with the hypha, but became well resolved in the latter 8-19 µm. This band is primarily associated with fatty acids and esters but is also associated with lignin (Heraud, Caine et al. 2007). The aldehyde and ketone peak (1729-1713 cm\(^{-1}\)) was only clearly resolved in the 8-13 µm spectrum, which was measured from the interface between the hypha and the xylem. This is an indication of lignin degradation in this narrow zone.

Figure 7.15: Spectra extracted from a segment of the S-FTIR map of the 2 day aquatically decomposed sample, shown here at the left, with z-values representing absorbance in the amide I band. Each spectrum represents a pixel with an area of 5 x 5 µm, which overlaps the adjacent pixel by 3 µm, to give a total transect length of 19 µm. Spectra are presented in a colour similar to that shown in the S-FTIR map. Pink - orange spectra are derived from the fungal tissue, blue spectra are derived from xylem and the green spectra are derived from the interface between these two tissue types. Chemical gradients radiating away from fungal tissues can be observed for polysaccharides and lignin. Protein is evident as amide peaks 1 and 2 in the first 2 spectra (pink and red). Carbonyl peaks indicating aldehydes & ketones and lipids (and lignin) are also illustrated.
7.3.6 Differentiating between different protein structures using FT-IR

The second derivative of the amide I peak can be used to reveal structural features of proteins (Byler and Susi 1986). In plant material the amide I peak has its apex at 1650 cm\(^{-1}\) (the \(\alpha\)-helix position) (Heraud, Caine et al. 2007). In fungal tissues, the apex of this peak is typically at 1648-1626 cm\(^{-1}\) (Szeghalmi et al., 2006) depending on the species.

The results shown in Figure 7.16 indicate that protein structures differed between the fresh and partially decomposed leaves. In the fresh leaf, maxima were seen at 1671, 1665, 1655, 1645 and 1634 cm\(^{-1}\), while in a leaf decayed in the water for 2 days maxima were seen at 1671-1668, 1665, 1660, 1657, 1652-1650, 1644, 1641, 1637 and 1628 cm\(^{-1}\).

Peaks from the spectra of partially decayed leaves had greater variation between replicates than spectra from fresh leaves. PCA analysis of the derivatised spectra indicated that amide I spectra from fresh leaves were substantially different from spectra taken from partially decayed leaves (Figure 7.17). The loadings for each principal component are shown in Table 7.2. This table indicates that the first three principal components differ in terms of the absorbance frequency of the major protein structures.

![Figure 7.16](Image)

Figure 7.16: The second derivative of three replicate spectra from fresh leaves, and three spectra from leaves submerged in the wetland for 2 days. Only wave numbers corresponding to the amide I peak are shown. Peak maxima represent different structural features of proteins such as extended...
chains (low components) at 1624-1637 cm$^{-1}$, $\alpha$-helices at 1654 cm$^{-1}$, unstructured components at 1645 cm$^{-1}$, and turns and bends at 1663-1694 cm$^{-1}$. Peak broadening and shifts to higher frequencies indicate weaker hydrogen bonding in the molecule.

Figure 7.17: PCR analysis of extracted spectra. Spectra extracted from regions of the S-FTIR maps that had strong absorbances in the amide I band were normalised and smoothed. The second derivative of the absorbance within the amide I band was then taken, as this region is indicative of protein structure. Principle components analysis indicated significant differences in protein structure between the two sample groups.

Table 7.2: The 2$^{nd}$ derivative peaks that gave positive loadings for principal components 1, 2 and 3. The protein structure associated with these peaks, as reported by Byler and Susi (1986), is shown in the column labelled “Protein structure”.

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</tr>
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</table>
Evidence for the formation of protein complexes

In the fresh and leached leaf samples there were no areas where amide I absorbance was spatially correlated with absorbance due to aromatic rings and ether linkages (Figure 7.5, a-c, (i), (ii) and (iii)). In the floodplain aged leaf, absorbance in these bands was associated over the entire area of mid-vein xylem (Figure 7.5, d (i), (ii) and (iii)). In the river samples there was initially a weak association between the spatial distribution of amide I and aromatic absorbance, and but the strength of the association increased with decomposition time (Figure 7.13, a-e (i) & (ii)). In the wetland samples these bands had a strong spatial association throughout the aquatic decay period, even as amide I absorbance decreased over time (Figure 7.12, a-e (i) & (ii)).

In S-FTIR images, absorbance in the amide I band was spatially correlated with fungal hyphae (Figure 7.18, a, b, c and d (ii)) (although spatial resolution was not adequate to discriminate all parts the hyphae from the background in (Figure 7.18, b (ii)), while absorbance in the amide III band coincided spatially with fibre sheath cells in Figure 7.18, a & d (iii), but with fungal tissues in Figure 7.18, c (iii). Absorbance in the amide III band coinciding with fibre sheath cells may be due to the presence of inorganic and organic nitrates, nitramines, and conjugated nitro compounds which absorb at similar frequencies to the N-H bonds from proteins normally associated with this wave band (see Table 7.1). Low absorbance due to aromatics from these tissues (Figure 7.18, a & d (iv)) suggests these nitrogen compounds are not aromatic.

S-FTIR images show that high absorbance in the aromatic band was correlated with xylem (Figure 7.18, a (iv)), immediately adjacent to an area of moderate absorbance in the amide I band. A more detailed examination of a hypha (Figure 7.18, b (ii) & (iv)) again indicated that protein associated with the hypha was concentrated in the area immediately adjacent to aromatic compounds. A similar pattern was observed in leaf
material that had been submerged in the river for 2 days (Figure 7.18, c & d, (ii) & (iv)). In Figure 7.18, d (ii) only a small amount of protein was evident within the hypha while adjacent areas of the hypha absorbed strongly in the aromatic band. Aromatics also exhibited moderate absorbance in (lignified) fibre sheath cells.
Figure 7.18: Fine scale relationships between protein and lignin distribution

This figure shows a segment of the midvein (60*120 µm) (a) and a map focused on fungal tissue (26*26 µm) (b) taken from the floodplain aged leaf. Similar maps are also shown for the leaf that was submerged in the Kiewa.
River for 2 days ((c) (60*225 µm) and (d) (44*36 µm) respectively). Images shown include the bright field image of the sample (i) indicating the area in which the FTIR map was acquired, and S-FTIR maps for amide I (ii), amide III (iii) and aromatic bands (iv).
7.4 Discussion

7.4.1 Synchrotron infrared micro-spectroscopy

By combining Fourier transform infrared spectroscopy with high resolution microscopy, fungal organisms within a substrate can be chemically characterized in a spatially explicit context (Garidel and Boese 2007). However, the diameter of fungal hyphae is similar to the wavelength of infra-red light (2.5-12.5 µm), and the mass of material to be analysed is very low, and this can result in light scattering and weak signals (Kaminskyj, Jilkine et al. 2008). The use of the synchrotron light source addresses these issues (Carr 2001; Garidel and Boese 2007; Miller and Dumas 2006; Reffner, Martoglio et al. 1995), making it possible for the biochemical components of individual hyphae to be identified (Jilkine, Gough et al. 2008; Szeghalmi, Kaminskyj et al. 2007). FTIR microscopy has been used to differentiate between fungal and bacterial infections in animals (Erukhimovitch, Pavlov et al. 2005) and to detect phyto-pathogens (Erukhimovitch, Tsror et al. 2005). In pure cultures, bacteria and fungi, and true fungi and Stramenopiles can be distinguished spectrally (Erukhimovitch, Tsror et al. 2005). However, there have been no reports where spectra from these organisms were able to be distinguished in a complex natural matrix such as a decomposing leaf.

7.4.2 Fungal colonisation of *E. camaldulensis* leaves

Microscopic examination of leaf sections using a conventional bright field microscope has shown that *E. camaldulensis* leaves are colonised by fungi while on the floodplain. At this time, fungi attack both the mesophyll and the leaf vascular tissue, where they specifically target phloem cells. Fungal hyphae probably invade minor veins at the leaf’s periphery, where the fibre sheath is sparse or absent, and begin attacking phloem at that point. Having penetrated the vascular network, hyphae appear to extend along the interior of the vascular bundle and consume the phloem as they progress. At the same time, fungal hyphae attack vascular tissues from the mesophyll, encircling the vascular bundle and attacking cells in the bundle sheath and collenchyma.

On entering the aquatic phase, the aged leaf consists mainly of vascular tissues sandwiched between the epidermal layers. The fibre sheath and xylem are attacked at this time. The decay of fibre sheath cells proceeds more quickly than xylem degradation. Xylem is attacked from its periphery, with outer cells being degraded while inner cells remain unaltered. Xylem showed no significant structural changes prior to 60 days in the
wetland and 107 days in the river. The leaf epidermis also remained unaltered for at least 60 days after entering the water. It is likely that lipids present in the epidermal tissues until that time prevented microbial attack of these cells.

7.4.3 Aquatic break down of lignin in *E. camaldulensis* leaves

Chemical maps obtained from FPA-FTIR and S-FTIR have shown that polysaccharides are lost from the leaf mesophyll during the terrestrial phase as a result of leaching. This is consistent with previous studies where chemical changes were measured in decomposing leaves (Gallardo and Merino 1992; Serrano 1992; Suberkropp, Godshalk *et al.* 1976). Light microscopy showed that fungal tissues were present throughout the mesophyll tissue after terrestrial aging, while the tissues of the xylem, fibre sheath and epidermis are largely intact but surrounded by fungal hyphae. This shows that non-lignified plant tissues are consumed preferentially.

On entering the aquatic phase, the majority of polysaccharides available to microorganisms are those within the leaf vascular tissues, and these are extensively cross-linked to lignin. Polysaccharides continue to be lost from the mesophyll, and polysaccharide signals from this region of the leaf were increasingly associated with fungal cell walls. Jilkine *et al.* (2008) found a strong peak at 990 cm\(^{-1}\) is associated with trehalose in fungal cell walls, but this peak was not well resolved in the data from this work.

High absorbance in the polysaccharide band from leaf vascular bundles suggests that these tissues were rich in cellulose, and hemicellulose, while contributions from starches, pectins and nucleic acids could not be ruled out (Heraud *et al.*, 2007). The discrete wave bands for these individual polysaccharides were mapped over the tissues, but the spatial distribution of absorbance for each separate polysaccharide matched that of polysaccharides generally, with minor differences in absorptivity.

As the majority of polysaccharides remaining in a leaf when it enters the aquatic phase are cross-linked to lignin, lignin break down is a necessary first step before aquatic organisms can obtain energy. While mineralisation of lignin is possible in aquatic systems (Bergbauer, Moran *et al.* 1992; Moran, Benner *et al.* 1989), it is limited by oxygen availability (Freeman, Ostle *et al.* 2004) and proceeds very slowly under anoxic
conditions (Benner, Maccubbin et al. 1984). Floodplain wetlands commonly suffer from low dissolved oxygen (Baldwin and Mitchell 2000), particularly in warm weather (Ford, Boon et al. 2002), and wetland sediments are generally anoxic (Baldwin and Mitchell 2000). Thus, it would be expected that lignin breakdown would proceed more slowly in the wetland than in the river, or fail to proceed at all.

Both microscopic and FTIR data show that there is some degradation of lignified tissues in the aquatic phase, and that degradation follows different temporal patterns in rivers and wetlands. The spatial distribution of aldehydes, as illustrated in FPA-FTIR images, indicates that this activity is highest in the floodplain aged leaf, but continues after immersion in the wetland then declines to low levels after 60 days. This may be a response to decreasing concentrations of dissolved oxygen as temperatures increased, or depletion of exploitable substrate. The distribution of aldehydes in leaf samples submerged in the river shows that lignin degradation increases over time and continues to be active even after 107 days in the aquatic environment. While subject to the same temperature regime as the wetland, flow in the river continually incorporates oxygen into the water column, making it less likely to be limited by dissolved oxygen. It is clear that enzyme mediated lignin breakdown is occurring in the submerged leaf detritus, but fungal decay of lignified leaf material begins earlier in wetlands than in rivers.

The fungal enzymes responsible for the breakdown of lignin include laccase (phenol oxidase) and lignin peroxidase. Laccase oxidizes phenolic moieties within the lignin structure to firstly form phenolic radicals, then quinones. This reaction may proceed via a number of different pathways, but generally C-C and C-O bonds are cleaved in side chains, aromatic rings (Kawai et al, 1988), and between monomers (Harkin and Obst, 1974). Some organisms may only demethylate lignin side chains (Leonowicz et al, 2001). Lignin peroxidase requires the presence of hydrogen peroxide (generated by other enzymes) for its activity. It catalyses a variety of C-C and C-O bond cleaving reactions, including the oxidation of the alkyl side chains of lignin, cleavage of C-C bonds in the side-chains of monomers, oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) monomers to the related aldehydes and ketones, and hydroxylation of benzylic methylene groups (Tien and Kirk 1984). The products of lignin peroxidase degradation of natural lignin provide a substrate for laccase, and these enzymes commonly co-occur (Leonowicz et al, 2001).
The end products of lignin breakdown reactions are largely ketones, acids and aldehydes, including coniferyl aldehyde and sinapyl aldehyde. Vibrations from the C=O bond in these compounds may absorb at frequencies within the amide I band. For example, the carbonyl group in coniferyl aldehyde and sinapyl aldehyde absorb at 1661 cm\(^{-1}\) and 1655 cm\(^{-1}\), respectively (Pomar et al, 2002). These monomers are extremely unstable and may re-polymerise (Leonowicz et al, 1985), particularly in the presence of lignin peroxidase (Sarkanen et al, 1991).

Earlier studies of leaves and wood decomposing in aquatic environments have revealed increases in the absolute lignin content over the first 6-12 weeks of decomposition, with corresponding increases in nitrogen and protein contents (Suberkropp, Godshalk et al. 1976; Weiland and Guyonnet 2003). The re-polymerisation of lignin monomers provides a possible mechanism for the generation of “new” lignin. These new polymers may then be deposited on pre-existing lignin. This would account for minimal changes in overall spatial distribution of absorption in the aromatic band, while discrete correlation with a rigid cell wall decreases with time. However it would not account for increases in the mass of lignin in decomposing leaves.

Complexation of lignin breakdown products with enzyme proteins is a possible mechanism for perceived increases in the absolute lignin content of decaying leaves. The most probable location that these complexes would form is in areas where enzymes are being secreted, such as the periphery of the xylem, where absorbance in the amide I band is often most intense.

A well resolved peak at 1510-1515 cm\(^{-1}\) (aromatic skeletal vibrations) is a common feature of the spectra of lignin from a variety of sources (Boeriu, Bravo et al. 2004; Gosselink, Abächerli et al. 2004). The height of this peak (absorbance) in spectra from xylem tissues decreased with distance from the hyphae. A significant reduction in the intensity of this band between 8 and 10 µm from the hyphae is consistent with enzymatic depolymerisation of lignins (Buta, Zadrazil et al. 1989). This band is poorly resolved in spectra from fungal tissues, and this may be due to the presence of non-lignin aromatic compounds such as those found in fungal melanins (Saiz-Jimenez, Ortega-Calvo et al. 1995). Changes in intensity and frequency of the band at 1284-1185 cm\(^{-1}\) (ether linkages
in lignin) indicates reduced absorbance due to $\nu_s$(C-O-C) and increasing absorbance due to $\nu_s$(C-OH) (Michell 1992). This is consistent with lignin breakdown via enzymatic cleavage of the linkages between monomers, such as occurs with the action of fungal laccases and peroxidises (Leonowicz, Cho et al. 2001).

### 7.4.4 The formation of humic substances

Low level absorbance in the amide I band is likely to be due to either microbial extracellular enzymes, or aromatic products of lignin break down (such as aromatic aldehydes absorbing in the region 1710 - 1685 cm$^{-1}$ (Silverstein, Bassler et al. 1974)). Earlier studies (Bärlocher, Tibbo et al. 1989; Suberkropp, Godshalk et al. 1976) have shown that fungal extracellular enzymes form complexes with plant derived tannins and polyphenols. If this is occurring in submerged *E. camaldulensis* leaves, we should observe increased spatial correlation between absorbance in the aromatic and amide bands over time. As tannins and polyphenols are abundant in mature *E. camaldulensis* leaves (Cork and Pahl 1996), and extra-cellular enzyme activity has been shown to occur (see Chapter 4), it is probable that protein-soluble polyphenol complexes will form. However, a large proportion of soluble polyphenols are lost via leaching during the terrestrial phase (Serrano 1992), so concentrations of these complexes may be low. FPA-FTIR and S-FTIR data suggest that such complexes may form in narrow zones at the periphery of lignified plant structures.

Low level absorbance in the amide I band associated with lignin in wetland leaves, and the Day 107 river leaf, may alternatively be due to carbonyl vibrations from lignin decomposition products, such as quinones, other aromatic aldehydes and aromatic ketones, which absorb at similar frequencies (Silverstein, Bassler et al. 1974). S-FTIR shows that while amide peaks are present in spectra from fungal tissues, they are poorly resolved in the region between hyphae and xylem (the region where enzymes are likely to be active), and absent at distances of greater than 10 µm from the hypha. This suggests that the distribution of protein-polyphenol complexes and lignin decomposition by-products is patchy at small spatial scales.

The data show that amide III absorbance (1328-1290 cm$^{-1}$) correlates spatially with amide I and II in the fresh leaf, a strong indication of protein. In decomposed leaves amide III absorbance is associated with the fibre sheath and fungal tissues for the first
month. In the later stages of decay, this pattern continued in the river, while in the wetland amide III absorbance became associated with xylem. Rice (1982) suggests that nitrogen is associated with heterocyclic aromatic humic compounds in the later stages of decay. It is likely that these humic compounds would include aromatic amines, which show a strong C-N stretch absorption in 1342-1266 cm\(^{-1}\) (Silverstein, Bassler et al. 1974). Amide III absorbance associated with the fibre sheath is probably associated with the production of aromatic amines as a result of fungal activity, but it is also consistent with C-N ring vibrations of melanin in fungal cell walls (Saiz-Jimenez, Ortega-Calvo et al. 1995). At the periphery of the xylem it is plausible that absorbance in this band is associated with aromatic amines or protein complexes. However, where absorbance peaks in amide I and II bands are absent, C-N bonds indicated by absorbance in the amide III band are unlikely to be from protein.

The FPA-FTIR data show a weak yet constant spatial correlation between absorbance in the aromatic band and absorbance in the amide I band in wetland leaf samples. In samples from the river, high absorbances in these bands do not coincide spatially until 60 days, when rates of lignin degradation were higher. While this is consistent with the formation of complexes between proteins and leaf polyphenols as proposed by Suberkropp et al. (1976), a more controlled experiment with longer incubations times would be desirable before drawing conclusions.

In both the wetland and the river, areas of highest absorbance in the polysaccharide band were spatially correlated with lignified plant tissues, as indicated by absorbance in aromatic and ether linkage bands. In the wetland, polysaccharide absorbance in these regions decreased over time, while in the river, absorbance decreased between day 2 and day 15, then increased for the remainder of the experimental period. This correlates with patterns of lignin decay as revealed by absorbance from aldehydes, and suggests that C-O-C bonds in polysaccharides are becoming disencumbered from crossed-linked lignin, enabling greater vibration. For this reason, higher absorbance in the polysaccharide band in observed in the river samples after lignin degradation has been initiated. We know from chemical analysis of decomposing oak and hickory leaves in a stream that losses of cellulose and hemicellulose closely reflect total weight loss, and that the rate of their loss remains relatively constant (Suberkropp, Godshalk et al. 1976). We can therefore
conclude that aquatic fungal communities are limited in their capacity to degrade and consume polysaccharides in leaf vascular tissues by their ability to first degrade lignin.

The trends observed in the polysaccharide absorbance with increasing distance from fungal hyphae, i.e. lower absorbance and broader peaks closer to fungal tissue, are similar to those seen in the fungal decay of wood (Weiland and Guyonnet 2003). Lower absorbance is associated with the depolymerisation of compounds such as cellulose, hemicellulose and pectin, and peak broadening has been associated with changes in intermolecular hydrogen bonding (Hsu 1997). These results show that polysaccharides are being depolymerised, and are consistent with spectroscopic observations reported by Gierlinger et al. (2008).

### 7.4.5 Protein and nitrogen enrichment

Both fresh and decomposed leaves contain protein, as indicated by absorbance in the amide I band. The protein content of the fresh leaf was spatially correlated with mesophyll cells, while in partially decomposed leaves protein is spatially correlated with fungal tissues. The second derivative of the amide I peak was compared between these two sources using PCA, and showed that these proteins were structurally different. Further to this, derivative spectra from the fresh leaf were similar to each other, while spectra in decomposing leaves varied significantly spatially and over time. It is likely that this difference reflects the presence of a single dominant protein present in the fresh leaf’s palisade mesophyll cells (ribulose bis-phosphate carboxylase; Heraud et al., 2005), compared with a variety of different proteins associated with different species of fungi (Szeghalmi, Kaminskyj et al., 2007), and hyphae of different ages and functions (Jilkine, Gough et al., 2008; Kaminskyj, Jilkine et al., 2008). Thus, FPA-FTIR and S-FTIR spectral mapping has revealed that leaf protein, from the mesophyll and phloem, is lost when leaves age on the floodplain, and is replaced by protein with a markedly different structure.

As we know that submerged leaves exhibit increased protein and nitrogen content when colonised by aquatic fungi, it has been proposed that the source of this additional protein is fungal biomass. The fungal mycelium is composed primarily of polysaccharides (80-90%, Bartnicki-Garcia, 1968), but the cell wall has an outer layer that is primarily protein (Klis, Mol et al., 2002). We can therefore expect fungal tissue to absorb strongly in the
amide bands. The data presented here show that absorbance in the amide I band within the decomposing *E. camaldulensis* leaf is spatially correlated with fungal tissues after incubation in either the river or the wetland. However, we can not assume that all nitrogen within the decomposing leaf is in the form of protein (Odum, Kirk *et al*. 1979).

Throughout aquatic leaf decomposition, low level absorbance in the amide I band is spatially correlated with absorbance in the aromatic band and lignified plant tissues, while higher level absorbance is found adjacent to the margins of the lignin. This is consistent with the formation of protein – polyphenol complexes which later adhere to the lignified tissues. However, given that absorbance at frequencies within the amide I band is also characteristic of lignin breakdown products such as quinones and aromatic aldehydes, we can not conclude from these data that protein complexes have formed. If indeed the amide I band is indicating the presence of protein complexes, they are in very low concentrations. Therefore, these results neither confirm nor contradict assertions by Melillo *et al*. (1984), Odum *et al*. (1979) and Rice (1982) that a proportion of the leaf protein is comprised of abiotic protein complexes.

Previous studies using FT-IR have shown that the amide II band may be poorly resolved in mature hyphae, which have less protein than new growth, contain large vacuoles (Szeghalmi, Kaminskyj *et al*. 2007) and may have melanised cell walls (Kaminskyj, Jilkine *et al*. 2008; Martin, Haider *et al*. 1974; Zhong, Frases *et al*. 2008). This poor resolution may be due to the presence of non-proteinaceous compounds in the leaf matrix that have NH$_2$ or N-H bending vibrations. Such compounds might include chitin, chitosan, and primary and secondary amides, which absorb at similar frequencies to the amide III band. The amide II peak was poorly resolved in the decomposed leaf sections. As many of the hyphae observed via light microscopy were darkly coloured, and probably had some chitin in the cell wall, poor resolution might be expected in spectra spatially correlated with fungal hyphae. In areas of the leaf where fungal tissues were not present, such as the xylem, poor resolution was probably due to nitrogenous decomposition by-products within the leaf matrix.

Absorption in the amide III band results from (C-H) and (C-N) stretch vibrations and (N-H) bending vibrations. This suggests Absorbance in this band may provide an indication of the distribution of organic nitrogen within a decomposing leaf. In line with Rice’s
(1982) suggested mechanism for nitrogen accumulation in decomposing plant material, absorbance in this band was correlated with fungal protein in the early stages of aquatic decay, but that the strength of this correlation decreased with time.

The results show that the protein and nitrogen in *E. camaldulensis* leaves are largely confined to fungal tissues during the early phase of aquatic decomposition. As decomposition progresses nitrogen and protein are increasingly associated with lignified plant tissues. This tends to support the notion that, as the energy of the leaf substrate is depleted, protein is entrained within abiotic complexes, and nitrogen is incorporated into non-proteinaceous organic compounds. Fungal growth and metabolism provide a possible mechanism for these transformations. For example, nitrogen is incorporated into chitin and melanins. As the fungal tissues senesce, these cell wall components may be further degraded to structures similar to the refractory heterocyclic compounds typical of humic material. This suggests that aquatic saprobic fungi biologically synthesise the precursors to humic substances in aquatic environments.

### 7.4.6 The ecological significance of these findings

The physical environment in rivers and wetlands differs markedly at scales that are relevant to aquatic fungi. Even though the fungal community colonising submerged *E. camaldulensis* leaves does not differ between these environments, it is now evident that these communities utilise their substrate differently.

The dried and senesced leaves of *E. camaldulensis* represent a poor food source for aquatic fauna (Bunn, De Deckker *et al.* 1986; Canhoto, Bärlocher *et al.* 2002; Canhoto and Graça 1999; Glazebrook and Robertson 1999), and while terrestrial aging removes many substances that may deter feeding, it results in a food source that is composed of polysaccharides and little else. Of the polysaccharides remaining, most are cross-linked to lignin, which limits bio-availability. So when these leaves enter the aquatic phase, they are colonised by fungi with the enzymatic capacity to degrade lignified plant cell walls (Leonowicz *et al.* 2001). These fungi, together with the substrate, then comprise a more nutritious food resource to higher trophic levels (Bärlocher and Kendrick 1975; Chung and Suberkropp 2009).
Lignin decay commences immediately after leaf immersion in wetlands, but substantial decay of lignin may be delayed for up to 2 months in rivers. This may be because leaves submerged in the river accumulate a surface biofilm (Golladay and Sinsabaugh 1991), composed of fungi, bacteria, algae and other organisms (Burns and Ryder 2001a; Schulze and Walker 1997). This biofilm may generate bio-available carbon (Haack and McFeters 1982) that can be utilised more readily than lignified plant tissues. Under these circumstances the breakdown of lignin would not be necessary until carbon generated within the biofilm became limiting. In wetland environments where biofilm does not accumulate on the leaf surface, the decomposer community would be required to initiate lignin degradation at an earlier stage. Further experimental work would be required to test this hypothesis.

Fungal ligninases are instrumental in the formation and transformation of aquatic humic substances (Claus and Filip 1998). The fungal degradation of lignin leads to the formation of refractory organic compounds that are unavailable to higher trophic levels and become stored in wetland sediments. Fungal metabolic processes are also responsible for sequestering nitrogen from the water column and incorporating it into proteins, chitin and aromatic compounds. While these compounds may undergo transformations in food webs and by other decomposer organisms, they represent the precursors of aquatic humic substances.

We know that fungi tend to dominate the early stages of aquatic leaf decomposition in terms of biomass and carbon mineralisation (Baldy, Gessner et al. 1995). However, it is certain that bacteria also contribute to this process, and more so in floodplain wetlands than in rivers (Baldy, Chauvet et al. 2002). Bacteria become dominant on submerged leaves when particle size becomes small, but many of these species may not be capable of degrading plant structural polymers (Suberkropp and Klug 1976). Bacteria are also the dominant decomposer organisms where dissolved oxygen is absent, such as in wetland sediments (Boon and Mitchell 1995; Boon, Virtue et al. 1996). While this work focuses on fungi, we know that bacteria have the potential to make significant contributions to protein enrichment and the break down of polysaccharides and lignin in submerged leaves. It is important to quantify this contribution in future work.
7.5 Conclusions

We have seen in earlier chapters that the fungal community on submerged *E. camaldulensis* leaves differs from that found on other litter types, and has the potential to accumulate significant amounts of biomass in both rivers and wetlands. As these leaves represent an abundant carbon resource in Australian floodplain environments, fungal breakdown of structural polymers and protein enrichment is a vital process connecting riparian plant production to higher trophic levels. This work demonstrates that *E. camaldulensis* leaves submerged in wetlands exhibit lignin degradation earlier than those in rivers, and that this may be due to more limited bio-available carbon in wetland benthic habitats.

Infrared mapping of leaves colonised by micro-organisms provides hitherto unseen spatially explicit insights into decomposition mechanisms, and supports earlier theories regarding nitrogen, protein and lignin enrichment of submerged leaves. Chemical mapping using Synchrotron and Focal Plane Array FTIR micro-spectroscopy has demonstrated that some microbial protein and other nitrogenous compounds may become complexed with polyphenols, or incorporated into heterocyclic aromatic compounds which are not bio-available. For this reason, C:N ratios may over-estimate the protein content of the leaf.

The high spatial resolution of this instrument has enabled us to detect the transformation of structural carbohydrates in plant vascular tissues into bio-available sugars as a result of fungal enzyme activity. Furthermore, it has provided evidence supporting the theory that lignin breakdown products released via oxidative enzyme activity form stable and refractory complexes within the decomposing leaf, and humic precursor compounds which may be stored in wetland sediments.

Given the limited replication in this exploratory research, further work is now required to quantify the importance of these processes. For example, it would be desirable to isolate the fungal species responsible for aquatic lignin degradation on submerged *E. camaldulensis* leaves, and re-introduce them to sterilised leaves. This would allow the contribution of fungi to leaf break down to be assessed independently to that of bacteria. Spatially explicit chemical analysis of such leaves using S-FTIR would benefit from concurrent mass spectroscopy or NMR analysis, to track the chemical changes produced
by fungal colonisation. This may enable us to characterise “new” lignin polymers. Further investigation of protein enrichment, carbohydrate breakdown, leaf biofilm dynamics and humic substance formation in *E. camaldulensis* leaves by fungi using $^{15}$N and $^{13}$C signatures may also be of benefit.
Chapter 8

General Discussion

8.1 Carbon Cycling in Australian Floodplain Wetlands

Our knowledge of the role of fungi in wetland carbon cycles has previously relied upon research performed outside Australia (Shearer, Descals et al. 2007). This research has established an understanding of fungal metabolism, explored taxonomic diversity and phylogenetic linkages between fungal groups, and investigated the ecology of aquatic fungi. While it is possible to apply this knowledge in Australian settings, the uniqueness of this country’s vegetation and climatic conditions demands that fungal communities in Australian floodplain wetland systems be investigated directly.

The exchange of carbon and nutrients between the river channel and the floodplain (including its wetlands) is discussed in the Flood Pulse Concept (Junk, Bayley et al. 1989), as is the adaptation of floodplain biota to periodic inundation. Fungi contribute to this exchange by incorporating the carbon from plant detritus, produced on the floodplain and in floodplain wetlands, into their own biomass. The propagules released into the water column are then distributed by the river channels and fungal mycelia and propagules may then be consumed by stream biota. Fungal activity may also facilitate leaching from submerged detritus. In arid systems, fungi are instrumental in releasing nutrients from stored biomass on the floodplain for use by plants, and their activities are synchronised with moisture availability. This has been described as the Pulse-Reserve paradigm (Collins, Sinsabaugh et al. 2008) and the flow of carbon through the system as the “funga loop”. These concepts are similar in approach, but deal with different magnitudes of moisture pulses, and different spatial scales of carbon movement. In the absence of a flood event, the movement of carbon stored on floodplains in arid environments is quite restricted.

Carbon dynamics in Australian floodplain wetland systems have been investigated on a number of fronts. These include the degradation of sediment carbon by bacteria, the break-down of submerged plant material, detrital food chains and pelagic food webs. An understanding of fungal processes and functions within this floodplain wetland ecosystem can now be integrated into this body of knowledge.
8.1.1 Carbon cycling in the sediments

In ephemeral floodplain wetlands a thin layer of oxic sediments overlies anoxic sediments. The boundary between these layers varies in both space and time as the wetlands flood and dry (Boon 2006). Rapid shifts to anoxic conditions are common (Boon 2000). Under these conditions, sediment carbon is mainly metabolised by methanogenic bacteria (Boon 2000), releasing methane and carbon dioxide firstly into the water column and then to the atmosphere, and the rate of methane emission increases with temperature (Boon, Mitchell *et al.* 1997). Aerobic decay of sediment carbon is considered to be a secondary source of carbon dioxide emissions.

In Chapter 5, it was shown that wetland sediments contain little fungal biomass, supporting the findings of Boon *et al.* (1996). Fungi do not appear to utilise sediment carbon, but lay dormant ready to utilise available substrates should conditions become suitable. Under the observed conditions, the impact of sediment fungi upon wetland carbon cycles is likely to be small.

8.1.2 The decomposition of submerged and standing dead plant material

While autochthonous productions in wetlands due to the growth of macrophytes and phytoplankton can be very high (Bott, Barnes *et al.* 1983; Briggs and Maher 1985; Roberts and Ganf 1986), significant amounts of organic material are also derived from allochthonous sources such as riparian vegetation (Briggs and Maher 1985; Campbell, James *et al.* 1992a). This production is occasionally consumed by grazers but more commonly decays *in situ*, either in the standing dead position or after entering the aquatic phase (Buesing and Gessner 2006; Kuehn and Suberkropp 1998a). Where the water column is oxygenated, decomposition may proceed quickly, but under anoxic conditions decomposition is slow and organic matter may begin to accumulate in the sediments (McLatchy and Reddy 1998).

In forested floodplain wetlands on Australian lowland rivers, the breakdown and decomposition of allochthonous plant material (eucalyptus leaves) has been shown to take around 4 months (Briggs and Maher 1983), with the latter half of this period being dominated by fragmentation (Glazebrook 1995; Janssen and Walker 1999). I have shown that mass loss in the earlier phase of aquatic plant litter decomposition is temporally correlated with high fungal biomass (Chapters 4 and 6).
During aerobic decomposition, complex carbohydrate polymers are cleaved by the extracellular enzymes of micro-organisms into monomers such as glucose, which can then be absorbed and metabolised (Chamier 1985). This thesis shows that material submerged in Australian floodplain wetlands is colonised by fungi, and that the biomass, structure and enzyme activity of the fungal community changes over time. Fungi have been characterised as the most important group of micro-organisms responsible for the aerobic decomposition of plant material in agricultural soils, forest soils, rivers and peat lands (Baldy, Gessner et al. 1995; Cromack and Caldwell 1992; Moorhead and Reynolds 1992; Thormann 2006), and it is now clear that they also degrade plant material in Australian floodplain wetlands.

8.1.3 Detrital food chains

Food chains represent the dominant pathway for carbon transformation in many ecosystems. Bunn and Boon (1993) investigated food webs in Australian floodplain wetlands and identified Cherax sp. as a major consumer of detrital carbon. They found that consumer groups were consuming a relatively $^{13}$C-depleted resource as well as riparian and macrophyte vegetation. Methanotrophic bacteria and planktonic Chlorophyta were suggested as candidates for the $^{13}$C-depleted carbon source. Fungi colonising submerged plant litter may also be candidates (Henn, Gleixner et al. 2002), but tend to have $\delta^{13}$C signatures similar to their substrates (Reid, Quinn et al. 2008). The $\delta^{13}$C signatures of Oomycetes colonising submerged plant detritus has not been determined in Australian systems, so this group is another potentially $^{13}$C-depleted resource.

The fungi that colonise submerged plant litter are an important food source to invertebrates such as caddisfly larvae (Cargill, Cummins et al. 1985), and provide elements of their diet essential for growth and reproduction (Albariño, Villanueva et al. 2008). Colonisation of submerged allochthonous litter by aquatic fungi and Oomycetes also results in a temporary increase in the leaf C:N, improving their food value (Chapter 6). The absence of a terrestrial aging period in litter decomposition prior to inundation, as may occur with summer flooding, may lead to lower levels of fungal colonisation and consequently reduced food resources for emerging invertebrates. In rivers, the observed increases in C:N in submerged leaves are larger (Chapter 6), as is the abundance and
diversity of shredder invertebrates relative to floodplain wetlands (Boulton and Lloyd 1991).

### 8.1.4 Pelagic food webs and phytoplankton decomposition

Carbon dissolved in the water column is utilised mainly by planktonic bacteria (Findlay and Sinsabaugh 1999). Large amounts of organic carbon are leached from *E. camaldulensis* leaves when they are submerged, and up to 50% of this is rapidly utilised by planktonic bacteria (Baldwin 1999). Extracellular enzyme activity provides further evidence that bacteria use much of the DOC in the water column of Australian floodplain wetlands (Boon 1989; Boon 1990; Boon and Sorrell 1991; Scholz and Boon 1993a; Scholz and Boon 1993b). In terms of carbon cycling, bacterial activity in these wetlands has been found to be consistent with the microbial loop concept (Pomeroy and Wiebe 1988). It should be noted that only weak linkages between grazing food webs and the microbial loop have been established (Thorp and Delong, 2002).

In the water column, the main fungal species are zoosporic fungi belonging to the Oomycetes and Chytridiomycota, and their motile propagules (Voronin 2008). The Chytrids are commonly parasitic on algae (Ellen Van Donk 1983; Kagami, Van Donk et al. 2004; Lopez-Llorca and Hernandez 1996) and Kagami *et al.* (2007) described the food chain whereby fungal parasites make algal carbon available to *Daphnia* as the “Mycoloop”. The Oomycetes are opportunistic and may colonise either living or dead organic material (Czeczuga, Godlewska *et al.* 2004; Czeczuga, Kozlowska *et al.* 2002; Czeczuga, Mazalska *et al.* 2005), often infecting physiologically stressed fish. The fungal community in the sediments may be important as a “seed bank”, storing encysted propagules of zoosporic fungi until suitable substrates and conditions are present in the wetland (Chapter 5). Thus a planktonic fungal community may be important to food webs involving cladocerans, diatoms and zoosporic fungi, and may influence the magnitude of algal detritus that accumulates in wetland sediments.

### 8.2 The Importance of Fungi in Australian Floodplain Wetlands

Gessner *et al.* (2007) outlined the conditions required to prove that fungi are important decomposer organisms in a given environment. These conditions are as follows:

1. Fungi must be present;
2. Fungi must be able to grow and reproduce under the prevailing conditions;
3. Fungi must produce the enzymes necessary to degrade the tissues of local plants;
4. Fungal activities should result in mass loss from organic matter; and
5. Fungi should compete successfully with other organisms in the system.

This study has shown that fungi are present on a variety of substrates in a floodplain wetland in the Murray-Darling Basin (Chapters 3). Fungal propagules from wetland water and sediments are able to colonise submerged *E. camaldulensis* leaves, grow, produce enzymes that break down the leaf components, and induce losses of mass and organic matter from the leaves (Chapter 4). Fungi are present in submerged wetland sediments (Chapters 3 & 5), but biomass is extremely low and there is no evidence so far that fungi either grow or utilise sediment carbon resources. On *E. camaldulensis* leaves, fungi compete with aquatic Oomycetes for resources, and both groups accumulate high biomass at some time during the leaf decomposition process. In order to prove that fungi were important decomposers of plant detritus in these floodplain wetlands, it would be necessary to demonstrate their ability to successfully reproduce.

The fungal communities in wetlands were observed to differ spatially within a wetland (Chapter 3), but not between three different wetlands (Chapter 6). The spatial differences observed in the pilot study may be due to stands of macrophytes and zones where allochthonous litter is deposited and the associated substrate specific fungal communities. In Chapter 6, only one substrate type was used and no spatial differences were observed. However, Oomycete community structure was observed to differ between wetlands on the same day (Chapter 6), indicating that the composition of these communities is influenced by factors in addition to substrate type.

There is evidence to suggest that fungal community structure is influenced by moisture content of drying wetland sediments (Chapters 3 and 5), and that these communities may be primarily composed of chytrid and Oomycete propagules and fungi that parasitise diatoms (Chapter 5). This community is unlikely to be active after extended periods of inundation due to low oxygen availability, or when sediments are exposed and dry, due to low moisture availability. The sediment moisture content and sediment inundation also influence the fungal biomass and enzyme activity on *E. camaldulensis* leaves lying on the sediment surface (Chapter 4).
When leaves age on the floodplain, their chemical composition is altered when compared with fresh leaves (Chapter 7), and these changes promote fungal colonisation (Chapter 4). However, the amount of organic matter lost from the submerged leaves does not differ between these leaf types. High resolution FT-IR spectro-microscopy (Chapter 7) has shown that polysaccharides and proteins in the leaf mesophyll are consumed rapidly, and this is followed by the simultaneous degradation of polysaccharides and lignin in the leaf’s vascular tissues. Lignin degradation begins sooner after inundation in wetlands than in rivers, and oils and waxes in the leaf epidermis are degraded more slowly than other leaf components.

8.3 Additional Observations

8.3.1 Fungal and Oomycete dynamics in rivers and wetlands

At sites where river flow was declining and wetlands were drying, Oomycetes biomass was high when the C:N of leaf material was high and fungal biomass was low. This suggests that the Oomycetes may be more important in improving the food quality of submerged plant detritus under warm, low flow conditions. Thus, estimates of fungal biomass based on ergosterol concentration only may underestimate the food value of conditioned *E. camaldulensis* leaves under these conditions. As the Oomycetes community structure differs between rivers and wetlands, and between sites, it is probable that flow and water quality are driving these differences.

Based on the observations made throughout this thesis, I suggest that a pattern exists in the way Oomycete and fungal organisms colonise leaf substrates in rivers and wetlands (Figure 8.1). This pattern consists of three phases (roman numerals), which may vary in duration. Leaves fall from a tree and may experience a period of decomposition on the floodplain prior to entering the water. Then, in phase I, a peak in biomass occurs immediately after immersion, comprised mainly of a few species of Oomycetes that exhibit strong growth and decline quickly. In phase II, the Eumycota become established, reach a peak in biomass and then decline. In this study, maximum biomass in this phase was higher in rivers than in wetlands and endured over a longer period. Phase III occurs after a disturbance, inducing stress. This disturbance may be high temperatures, low flow, low oxygen availability, wetland drying or declining substrate quality. In this phase, we see an increase in Oomycete biomass followed by a decline, while fungal biomass remains stable but at low levels.
This pattern in biomass accumulation can be related to the movement of the leaf substrate through the wetland and floodplain environment, and the spatial distribution of various types of fungal and Oomycete propagules throughout the environment. Whilst on the tree, or during floodplain aging, leaves may attract the propagules of plant pathogens and litter and soil fungi. There are many species of true fungi and Oomycetes in both these groups. When the leaves enter the aquatic phase, they tend to float on the surface for some time (personal observation). At this point, Oomycetes begin to grow, and accumulate a large amount of biomass that also declines quickly. The leaf may also encounter the propagules of aquatic and aero-aquatic hyphomycetes that float on the surface of the water. The partially decomposed leaf then begins to sink through the water column. Here, aquatic and aero-aquatic hyphomycetes begin to accumulate biomass, and the leaf may encounter the propagules of zoosporic fungi. As the substrate quality and the availability of oxygen declines, the fungal biomass also declines, until the leaf settles on the sediment surface. If a disturbance such as wetland drying or periodic exposure due to low river flows occurs, an increase in Oomycete biomass may be seen. The leaf may encounter yeasts and dormant propagules in the sediments that are able to utilise the remaining leaf resources should the wetland remain dry long enough for these fungi to become established. The time the leaf spends in each of these phases is currently unknown, but is likely to be extremely variable.

Members of the Oomycetes are able to secrete enzymes that degrade lipids (Beakes and Ford 1983; Mozaffar and Weete 1993; Rand and Munden 1992), enabling them to colonise the waxy surface of the *Eucalyptus* leaf, and infect living fish. This may explain their abundance in phase I. Once this layer is breached, the Eumycota are able to colonise the leaf mesophyll, and deter further presence of Oomycetes through competition for resources or by producing chemical inhibitors (Fryar, Booth *et al.* 2005; Mille-Lindblom, Fischer *et al.* 2006a). When high quality food resources in the leaf become diminished, and dissolved oxygen levels are reduced, a different group of Oomycetes may then compete successfully for use of the remaining resources. Increased lignin breakdown is observed during phase III in streams.

The molecular methods used to compare fungal communities are sensitive, and reveal the full diversity of the community. However, they are not able to distinguish between active
fungal organisms and resistant propagules. In the case of mycorrhizal communities in a pine forest, Taylor and Bruns (1999) found only 10% of the species comprising resistant propagules were found actively colonising plant roots. Thus we should attempt to discriminate between active and dormant fungal species before making inferences about the impacts of changes in fungal community structure upon ecosystem functioning in general, and carbon cycling specifically.
Figure 8.1: A schematic diagram representing the temporal patterns observed when a leaf substrate is colonised by fungi and Oomycetes (top) and an explanation for this temporal pattern in terms of the spatial distribution of fungal propagules within the wetland and floodplain ecosystem (bottom).
Time periods are indicated by roman numerals, indicating the biomass accumulating on the substrate (top), and the likely position of the substrate when these organisms colonise and grow to maximum biomass levels (bottom).

8.3.2 The decomposition of lignin by aquatic fungi
In Chapter 4 it was observed that oxidative enzyme activity on *E. camaldulensis* leaves was higher under aquatic conditions than under floodplain conditions. This suggested that lignin decomposition was occurring in submerged leaves. Hydrolytic enzyme activity showed that polysaccharides were being degraded at the same time. These chemical changes were examined in Chapter 7, and it was shown that *E. camaldulensis* leaves submerged in wetlands exhibit lignin degradation earlier than those in rivers. This may be due to more limited bio-available carbon in wetland benthic habitats (Chapter 7).

Chemical mapping using Synchrotron- and FPA-FTIR micro-spectroscopy revealed hitherto-unseen spatially-explicit insights into decomposition mechanisms in leaves colonised by micro-organisms, and provided evidence to support earlier theories that lignin breakdown products released via oxidative enzyme activity form stable and refractory complexes within the decomposing leaf. Microbial protein and other nitrogenous compounds appear to become complexed with polyphenols, or incorporated into heterocyclic aromatic compounds which are not bio-available. Energy for lignin degradation may be derived from the enzymatic break-down of bio-available sugars, which occurs simultaneously with lignin degradation.

8.3.3 Issues of scale
During an ecosystem disturbance such as flooding or drying it is highly probably that the biomass and diversity of the fungal community will be altered, but the effect on different species and individual organisms will vary between physical locations and with disturbances of different types and magnitudes (Morris, Friese *et al.* 2007). However, the spatial scale over which such a disturbance occurs differs from the scale at which the response of fungal communities was measured in this study.

Fungal communities are intrinsically patchy. Within a plant substrate such as a leaf, there is variability in the distribution of fungal species, and patchy distribution of plant detritus within a wetland leads to patchy distribution of fungal communities on the wetland scale. In addition, wetland ecosystems occur as patches within the floodplain landscape. Thus,
fungal ecology in wetlands lends itself to analysis in terms of patch dynamics models (Pattee and Chergui 1995; Pickett and White 1985; Townsend 1989). Under this paradigm, at the landscape scale major flood events may disperse fungal propagules allowing re-colonisation of disturbed sites. Ecosystem scale disturbances such as wetland drying would influence substrate availability, leading to changes in the biomass and structure of fungal communities on patch (e.g. collection of submerged plant debris, reed stand) and community (individual leaves) scales. At the same time, altered fungal community structure and function may influence change at patch and ecosystem levels. For example, increases in the abundance of opportunistic pathogens may limit phytoplankton productivity (Kagami, de Bruin et al. 2007), or loss of saprotrophic species may impact on nutrient availability and alter plant productivity (Hunt and Wall 2002).

Flooding and drying in floodplain wetlands are predictable disturbances, and fungal communities in these ecosystems will have developed resilience to these changes (Morris, Friese et al. 2007). That is, they have the capacity to return to a pre-disturbance state. However, river regulation and climate change have led to changes in the temporal pattern of these disturbances, reducing their predictability, and perhaps impacting upon the resilience of the fungal community.

The experimental findings in this thesis have investigated fungal dynamics at the community scale, and the inherent patchiness has often resulted in large variations about mean results. Changes in the structure and functioning of the fungal communities examined are likely to influence processes within the wetland ecosystem, but extrapolation of these findings to the ecosystem scale should be applied with extreme caution. A larger number of replicates would be needed at the patch and ecosystem scales before confident predictions could be made as to the effect of changes in the fungal community on the functioning of floodplain wetlands in general.

8.4 Knowledge Gaps

The work of Thomas et al. (1992) showed that fungal community structure differs between leaf substrates from different plant species. Evidence to suggest that this may also be true in wetlands was presented in Chapter 3. However, further work is required to show that fungal communities differ between the standing dead blades of different
species of aquatic macrophytes, and the submerged detritus of other wetland plants. The identity of fungal species parasitising phytoplankton and the role of fungi in decomposing dead algal cells in sediments also remain entirely unexplored in Australian wetlands.

The response of the fungal communities in Australian floodplain wetlands to environmental variables has not been investigated. These include their response to changes in temperature and season, and changes in water quality such as increased concentration of dissolved nutrients, salts and potentially toxic substances, and decreased oxygen concentrations. In particular, it would be an advantage to know whether the fungal communities in floodplain wetlands exhibit resilience to disturbances such as increased salinity, acidification and black water events.

It has been shown that leaf conditioning results in increased nitrogen content (Melillo, Aber et al. 1982; Suberkropp, Godshalk et al. 1976; Thornton 1965 and Chapter 6). However, we do not know which organisms are responsible for this process in wetlands, or the origin of the additional nitrogen. Research in river systems in the USA and Canada has shown that aquatic hyphomycetes are able to utilise dissolved nitrogen from the water column (Sridhar and Bärlocher 2000; Suberkropp and Chauvet 1995), but this may vary with the concentration of nitrogen both in the water column and in the substrate. Laboratory experiments under flowing and standing water conditions might utilise $^{15}$N and $^{13}$C enriched water to track the sources and sinks of nitrogen in decomposing plant detritus.

It would also be an advantage to determine what proportions of carbon loss from submerged leaves can be attributed to leaching and fungal production. Personal observation suggests that fresh *E. camaldulensis* leaves submerged in sterile water undergo virtually no leaching, and that some microbial activity is required to breach the waxy leaf cuticle and initiate the leaching processes. This needs to be confirmed experimentally.

While evidence from rivers (Chessman 1986; Davis and Winterbourne 1977) and laboratory experiments (Albariño, Villanueva et al. 2008; Bärlocher and Kendrick 1975; Graça, Maltby et al. 1993; Graça, Pozo et al. 2002) has shown that invertebrate shredders
consume aquatically conditioned leaves, it is not known whether shredders consume conditioned eucalypt leaves submerged in wetlands. Given that these leaves do not improve in food quality to the same extent as those in rivers, and that shredders are in low abundance in these systems, it is possible that these leaves are not consumed by shredders. In wetlands, it is possible that scraper organisms such as aquatic snails are consuming the fungal biomass from the surface of submerged leaves.

8.5 Future Directions

In this thesis, T-RFLP has been used to compare the community structure between samples of substrates. This method does not endeavour to identify the species involved. However, without knowledge of the species comprising the fungal community it is difficult to determine how environmental disturbances might influence the structure and function of the fungal community, or how changes in the fungal community might impact upon the wetland ecosystem. Therefore, future investigations into fungal ecology in wetlands should attempt to identify species colonising plant, phytoplankton and sediment substrates. This might be done using conventional culturing techniques to isolate and grow wetland fungi before describing their morphology and sequencing the DNA, or utilise the emerging metagenomics technologies that enable us to derive DNA sequences (usually 18S) of all organisms present within a sample.

The extraction of ergosterol from fungal biomass provides an efficient method for estimating fungal biomass on plant substrates, given some understanding of the composition of the fungal community. However, this method does not provide an estimate of Oomycetes biomass, and there is no similar method currently available that can do so with the ease and speed of the ergosterol method. As the cell wall sterols in the Oomycetes are also found in other aquatic organisms, molecular methods such as real-time quantitative PCR may provide the best options for developing a useful Oomycete biomass estimation method.

Time constraints did not allow concurrent measurement of extra-cellular enzyme activity and substrate chemical composition in river and wetland conditioned leaves (Chapter 6 and 7). The simultaneous measurement of these variables would have enabled an exploration of relationships between the metabolic processes of fungi and the breakdown of components of the leaf substrate. Samples have been retained and there is potential for
this work to be conducted in the future. Spatially explicit chemical analysis of partially decomposed leaves using S-FTIR would be further complemented by concurrent mass spectroscopy or NMR analysis, to track the chemical changes produced by fungal colonisation. This may enable the characterisation of “new” lignin polymers.

Further work is required to quantify the rate of breakdown of lignin, polysaccharides, lipids and proteins in decomposing leaves, and to link this to submerged detritus biomass estimates. This would allow conclusions to be drawn regarding the importance of these processes to wetland carbon cycling. In addition, the fungal species responsible for aquatic lignin degradation on submerged *E. camaldulensis* leaves should be isolated and identified, and the relative contribution of these organisms and bacteria should be determined.

### 8.6 Concluding Remarks

In floodplain wetlands, fungi are important in the breakdown of plant structural polymers, and in enriching detritus with protein. In this role, they form a vital link between plant production and higher trophic levels, and may be instrumental in the formation of aquatic humic substances. As a field of enquiry, the study of the ecology of aquatic fungi in floodplain wetlands is in its infancy. There is also ample opportunity for exploration of fungal taxonomy and ecophyloogy. A range of emerging spectroscopic, molecular and isotopic techniques could possibly be applied to explore these areas to improve our understanding of wetland ecosystem nutrient cycles and biotic interactions.
Appendix 1: Optimising Noise Reduction

The affect of different peak area threshold values on data richness is illustrated in Figure 8.2. The optimum threshold value is indicated by the data point immediately following an initial change in slope of the data series. The graph indicates that optimum noise reduction lies between 0.1 and 0.5% for most samples. Therefore, data analysis was performed using a noise reduction of 0.25%.

Figure 8.2: This graph illustrates the number of data points remaining for each T-RFLP sample after noise was removed. The percentage of noise reduction is shown on the x-axis, while the number of taxonomic units represented is shown on the y-axis.
References


Bärlocher F (1992) 'The ecology of aquatic hyphomycetes.' (Springer-Verlag) 225


Boon PI (1990) Organic matter degradation and nutrient regeneration in Australian freshwaters: II. Spatial and temporal variation and relation with environmental conditions. Archiv für Hydrobiologie 117(4), 405-436. [In English]


257

Boundy I Histological Methods. In 'DEV2011: Early human development from cells to tissues.' pp. 6. (Monash University, Department of Anatomy and Developmental Biology)


Bunn SE (1988b) Processing of leaf litter in two northern jarrah forest streams, Western Australia: II. The role of macroinvertebrates and the influence of soluble polyphenols and inorganic sediment. *Hydrobiologia* 162, 211-223. [In English]


Convention on Wetlands (Ramsar I, 1971) Classification System for Wetland Type. In 'Key Documents of the Ramsar Convention.'


Dick MW (1976b) The ecology of the aquatic phycomyces. In 'Recent advances in aquatic mycology.' Ed. EBG Jones). (Elek Science: London)


Elton CS (1927) 'Animal ecology.' (Sidgwick & Jackson, LTD.: London) 207


England JR (2001) Changes in the morphology and anatomy of leaves and sapwood of *Eucalyptus regnans* F. Muell. with age., University of Melbourne, Melbourne


Erickson K-EL, Blanchette RA, Ander P (1990b) 'Microbial and enzymatic degradation of wood and wood components.' First edn. (Springer-Verlag: Berlin) 407


Esau K (1977) 'Anatomy of seed plants.' 2nd edn. (John Wiley and Sons: Brisbane)


Evert RF, Esau K (2006) 'Esau's Plant anatomy: meristems, cells, and tissues of the plant body : their structure, function, and development.' (John Wiley and Sons: Hoboken, New Jersey) 601


Glazebrook HS (1995) The effect of floods on leaf litter breakdown rates and nutrient dynamics in a river red gum (*Eucalyptus camaldulensis* Dehnh.) forest. Charles Sturt University, Faculty of Science and Agriculture, School of Environmental and Information Sciences.,


Gribben DL (2000) Norman's Lagoon: an examination of its phytoplankton and photosynthetic bacterial populations, and the physico-chemical factors influencing them., La Trobe University, Wodonga

Griffin DM (1972) 'Ecology of Soil Fungi.' (Chapman and Hall: London)


271


Johnson TWJ, Seymour RL, Padgett DE (2002) 'The biology and systematics of the Saprolegniaceae.' (Ilumina: Educational Resources for Science and Mathematics Published online)


278

Lund A (1934) Studies on Danish freshwater phycomycetes and notes on their occurrence particularly relative to the hydrogen ion concentration of the water. Det Kongelige Danske Videnskabernes Selskabs Naturvidenskabelige og Matematiske Afhandlinger 9(6), 1-97.


Merritt RW, Cummins KW (1978) 'An introduction to the aquatic insects of North America.' (Kendall/Hunt Publishing Company: Dubuque, Iowa) 441


Schoenlein-Crusius IH, Pires-Zottarelli CLA, Milanez AI, Humphreys RD (1999) Interaction between the mineral content and the occurrence number of aquatic fungi in leaves submerged in a stream in the Atlantic rainforest, São Paulo, Brazil. *Revista Brasileira de Botânica* 22, 133-139.


Silvar C, Diaz J, Merino F (2005 ) Real-time polymerase chain reaction quantification of *Phytophthora capsici* in different pepper genotype *Phytopathology* **95**(12), 1423-1429. [In English ]


Suter SGA (2009) Aquatic fungi in an alpine stream of south-eastern Australia Honours Thesis, La Trobe University, Albury-Wodonga

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