

MICROBIAL INTERACTIONS ON DECOMPOSING SPARTINA ALTERNIFLORA:
USE OF FUNGALLY-MODIFIED LEACHATE BY BACTERIAL COMMUNITIES IN AN
EXPERIMENTAL SALT MARSH DECOMPOSITION SYSTEM

by

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(Under the Direction of James T. Hollibaugh)

ABSTRACT

Spartina alterniflora decomposition is an important route for carbon transfer to estuarine detrital food webs. The two major microbial groups of decomposers are fungi and bacteria, both studied separately in the *Spartina* decay system. By using flow-through microcosms, we studied how fungi affect *Spartina* decomposition products and how bacteria respond to the addition of these products as growth substrates. We found that fungi do not significantly alter leaf characteristics over < 40 day incubations, but fungi decrease *Spartina* particle removal and immobilize leached dissolved organic carbon (DOC), nitrogen, and phosphorus. Bacteria grown on these fungally-modified leachates did not respond numerically. However, bacterial operational taxonomic unit (OTU) diversity and ribotype diversity decreased with fungal influence. Leachates from fungally-modified *Spartina* leaves enhanced the relative proportion of alpha-Proteobacteria, the major *Spartina*-associated ribotype in nature. Overall, however, there was no conclusive evidence for antagonism between fungi and bacteria in the *Spartina* decay system.

INDEX WORDS: *Spartina alterniflora*, decomposition, DOC, leachate, chitinase, fungi, bacteria, DGGE

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DEDICATION

For their ceaseless love, encouragement, and patience, I dedicate this thesis to my mother and father: Theresa and David Biers.

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CHAPTER 1

INTRODUCTION

I. Salt Marsh Ecology

A. Global Importance of Salt Marshes

Salt marsh ecosystems, transitions between terrestrial and aquatic habitats, are globally important for their economic and ecological value. Because salt marsh plants help stabilize the soil, marshes are effective in erosion moderation. In addition, they improve water quality by retaining pollutants and excess nutrients. Ecologically, they provide habitat for numerous species of organisms, including many that are commercially important.

The “outwelling hypothesis” posits that organic matter exported from coastal marshes fuels food chains in adjacent sea waters (Teal 1962; Odum 1968; Nixon 1980; Lefeuve and Dame 1994). Material such as dissolved nitrogen is also transported from marshes to coastal water and can account for over 40 % of the dissolved nitrogen in the near-shore water column (Valiela 1984). Freshwater marsh ecosystems exhibit this nutrient transfer as well. For example, most of the organic matter in the Amazon River consists of vascular plant remains of marsh origin (Hedges et al. 1994). This nutrient and organic matter transfer creates a significant link for nutrients between marshes and adjacent bodies of water.

Salt marshes such as those along the North American Atlantic coast are highly productive (estimated to be $2,840 \text{ g} \cdot \text{m}^{-2} \cdot \text{y}^{-1}$) (Schubauer and Hopkins 1984). Primary producers in these systems include benthic and epiphytic microalgae, phytoplankton, benthic macroalgae and vascular plants. The dominant primary producers in salt marshes, vascular plants, represent a large component of living biomass; they also play a major role in the global carbon cycle (Olson et al. 1985). As vascular plants decay, their detritus becomes a large and renewable pool of organic material. Benthic microalgae are also important primary producers in salt marshes. Several studies suggest that benthic microalgae produce at least 40 % of the aboveground primary production of common vascular plants in east coast U.S. marshes and possibly west coast U.S. marshes as well (Zedler 1980; Sullivan and Moncreiff 1988; Pinckney and Zingmark 1993; Newell and Porter 2000). Because of their productivity, benthic microalgae have been studied for their importance to marsh foodwebs (Pomeroy et al. 1981; Fry and Sherr 1984; Sullivan and Moncreiff 1990).

B. Dominance of *Spartina*

The dominant vascular plant in eastern Atlantic coast marshes of the U.S.A. is smooth cordgrass (*Spartina alterniflora* Loisel). Estimates of primary production for *Spartina* in Georgia marshes range from 1,100 to 7,600 $\text{g dry mass} \cdot \text{m}^{-2} \cdot \text{yr}^{-1}$ (Schubauer and Hopkins 1984). In Massachusetts' Great Sippewissett Marsh (GSM), Teal and Howes (1996) measured average annual biomass production of various *S. alterniflora* forms. Aboveground, short form *S. alterniflora* produced from $370\text{-}501 \text{ g} \cdot \text{m}^{-2}$ annually while creek bank forms produced from $1,023\text{-}1,623 \text{ g} \cdot \text{m}^{-2}$; belowground *S. alterniflora* produced $872\text{-}1,021 \text{ g C} \cdot \text{m}^{-2}$ annually. Overall, *S. alterniflora* contributes approximately

81 % of the total primary production in east coast intertidal marshes (Pomeroy et al. 1981). Because of its dominance and productivity, *S. alterniflora* has been extensively studied (e.g. Keefe 1972; Turner 1976; Pomeroy et al. 1981; Marinucci 1982; Dardeau et al. 1992).

Carbohydrates are the most abundant constituents of vascular plant tissues because they provide structure (e.g. cellulose, hemicellulose and pectin) and storage (e.g. starch and sucrose); they are also peripheral or integral components of other major plant compounds such as lignin and tannin (Aspinall 1970; Sjostrom 1981; Tanner and Loewus 1981; Zucker 1983). Due to the high percentage of carbohydrates in vascular plant tissues, most carbon and energy flow resulting from *Spartina* decay directly involves carbohydrate oxidation (Opsahl and Benner 1999).

The main carbohydrate in *S. alterniflora* tissues is lignocellulose (LC), a biopolymer consisting of a more refractory (lignin) and a more labile (polysaccharide) component. About 75-85 % of living *S. alterniflora* dry weight is lignocellulose (Maccubbin and Hodson 1980; Hodson et al. 1982; Hodson et al. 1984; Benner et al. 1985) with the remainder containing starch and lipids. Percentages of LC content are even higher in senescent and standing-dead plants (Hodson et al. 1984). Since it is more labile, polysaccharide degradation rates are 3-7 times those of lignin degradation in lignocellulose (Benner et al. 1984a; Benner et al. 1984b; Benner et al. 1984c; Hodson et al. 1984; Bergbauer and Newell 1992). This results in lignocellulose detritus becoming enriched in carbon from lignin and therefore more recalcitrant. As a result, lignin contributes a greater portion of the carbon in detrital *S. alterniflora* than in living tissues (Wilson et al. 1986).

C. Breakdown of *Spartina*

When leaf blades of *S. alterniflora* cease photosynthesizing, they die in succession from the base of the plant toward the tip of the shoot (Hardisky 1980). Dead leaf blades are not abscised from the plant; rather, they remain upright and attached to the shoot as they undergo early stages of decay. These attached, dead leaf blades are hereafter referred to as standing dead leaves. When compared with live *S. alterniflora*, standing dead leaves comprise 65.5 % of the standing grass biomass (Schubauer and Hopkinson 1984). During later stages of decay, these leaves bend toward the sediment where they will further decompose. The majority of *S. alterniflora* above-ground biomass decomposes aurally rather than in a submerged state (Newell and Fallon 1989). Therefore, it is mostly detrital *S. alterniflora*, not living tissue, which enters the food web.

Because *Spartina* is so prevalent in temperate salt marshes and because dead leaves are not abscised from the stalk, the process of aerial decomposition is integral to marsh functioning. Microbes are the main decomposers of lignocellulose since LC is highly refractory, not easily leached, and inaccessible to most other organisms. By fueling microbial metabolism, decomposition of plant tissues becomes a major route for carbon remineralization in aquatic and terrestrial ecosystems (Martens et al. 1992; Moers et al. 1994).

The general processes of *Spartina* decay are similar to those for other non-living organic matter. Three phases of decay occur in nonliving organic matter: leaching of soluble compounds, degradation by microbes, and slow decay of refractory material (Kaushik and Hynes 1971; Gosselink and Kirby 1974; White et al. 1978; Haines and

Hanson 1979; Lee et al. 1980; Montagna and Ruber 1980; Marinucci et al. 1983; Valiela et al. 1985). All three processes—leaching, fast microbial degradation, and slow microbial degradation—take place throughout plant matter decay.

Leaching, usually considered a physical mechanism of decomposition, is a process that rapidly transports soluble material out of the plant. The length of this phase is typically short, lasting from minutes to weeks. For example, studies using *S. alterniflora* have leaching periods lasting from 21 – 42 days (e.g. Valiela et al. 1985; Opsahl and Benner 1999). Rates of plant decay during this phase are high with 5 to 40 % of litter lost (Cole 1982; Valiela et al. 1985). This decay is most likely not microbially mediated since it occurs at similar rates in sterile and nonsterile conditions (Cole 1982). The resulting leachate is available to heterotrophs for uptake and mineralization (Otsuki and Hanya 1972; Godshalk and Wetzel 1978a; Newell et al. 1981).

Microbial degradation occurs over longer periods of time—months to years—and produces lower rates of mass loss than leaching (Godshalk and Wetzel 1978b). An additional 40 to 70 % of the original plant material is lost in this manner. Compounds solubilized by enzymatic hydrolysis are taken up by the microbes and either respired as CO₂, solubilized into dissolved organic carbon (DOC), or incorporated into biomass. As a result, the production of labile microbial biomass provides a link for the transfer of carbon and energy from primary production to higher trophic levels (Odum and de la Cruz 1967).

Consumption of plant material by non-microbial heterotrophs has been studied in conjunction with microbial degradation because it is difficult to separate their activities. Metazoan herbivores and other heterotrophs also contribute to *Spartina* degradation.

For example, a common salt marsh snail, *Littoraria*, shreds *Spartina* plants (Bertness 1984). This snail ingests the plant and causes an estimated 2 to 3 % of plant organic mass to be lost per day (Newell and Barlocher 1993). Other important organisms contributing to *Spartina* degradation include various gastropods, arthropods, crustaceans, and insects. However, it has been estimated that less than 5 % of the living, green standing crop is grazed by herbivores (Smalley 1959; Teal 1962) due to unpalatable and refractory compounds. Microbial communities can further degrade shredded plant components.

Whatever the mechanism, after heterotrophic decomposition, the remaining detritus is enriched in highly refractory phenolic polymers and complexes (Nissenba and Kaplan 1972). These either remain within the marsh to degrade further or are transported into coastal waters. Decomposition of refractory compounds, the third phase of degradation, is very slow. However, by this stage, as little as 10 % of the starting plant material remains.

For the purpose of this study, two factors controlling *Spartina* decomposition are discussed further. First, leaching rates and components are described in greater detail. Second, the process of microbially mediated plant decomposition is discussed. In particular, the two principle microbial competitors for organic substrate decomposition, bacteria and fungi, are characterized.

II. Leaching: First Phase of Decomposition

A. General Properties of Leaching

The process of leaching has been studied extensively in plants (e.g. Tukey 1970; Godshalk and Wetzel 1978a; Turner 1978). Typical of aquatic plant decomposition

studies, initial rapid loss in mass occurs (e.g. Godshalk and Wetzel 1978a; Godshalk and Wetzel 1978b; Godshalk and Wetzel 1978c; Valiela et al. 1985). As plants begin to decompose, a substantial portion of organic matter is solubilized and rapidly leached from particulate material (Tukey 1970; Godshalk and Wetzel 1978a). Inorganic matter is released as well (Tukey 1970; Turner 1993; White and Howes 1994b; Gessner et al. 1999; Burke et al. 2000).

B. Factors Affecting Leaching Rates

Many factors can affect leaching rates. For example, Gallagher et al. (1976) proposed that variation in DOC release was due to temperature fluctuations, recent rainfall, frequency of tidal inundation, leaf age, and photosynthetic rates. In fact, Tukey (1970) found that more carbohydrates were leached at higher temperatures. The intensity and volume of leaf wetting is also a factor in leaching rates, for leaves only need to be wetted to begin leaching (Tukey 1970); the duration of the wetting episode impacts the leaching rates. Rainfall, dew accumulation, and tidal submergence are prominent routes for leaf wetting in the environment (Tukey 1970). When leaves are completely submerged, leaching rates are initially large then level off to rates that are about ten times that of nonsubmerged plants (Turner 1978). The salinity of the wetting agent may also affect leaching rates. Tukey (1970) found that sodium salts in rain droplets only slightly increase leaching rates. However, using litterbag experiments of submerged plants, Turner (1993) found that higher amounts of carbon were leached under higher salinity (30 ppt versus 10 ppt). Another factor affecting leaching rates is leaf integrity, for damaged cells can release their contents easily. In fact, Tukey (1970) found that young, actively growing plant tissue is less susceptible to leaching than older

tissue. All of these factors contribute to a seasonal peak in C, N, and P leaching during the summer when biomass is highest (Turner 1993).

C. What is Leached from Plants

Large mass losses incurred over the leaching phase are attributed to removal of easily extractable compounds such as cellular metabolites (Opsahl and Benner 1999). In order to determine the extent of leaching rates, Turner (1976) compared the rate of leaf leachate loss to *S. alterniflora* production in south Louisiana. He found that the total amount of carbon released by leaching ($200 \text{ g C}\cdot\text{m}^{-2}\cdot\text{yr}^{-1}$) represents 5-10 % of the total net aboveground plant production in that system. Comparable amounts and percentages have been measured for other ecosystems (Sieburth 1969; Sieburth and Jensen 1969; Newell et al. 1980), but lower percentages have been calculated by using radiocarbon rather than direct chemical measurement (e.g. Wetzel and Manny 1972; Brylinsky 1977).

D. Organic and Inorganic Leachate

Generally, more organic than inorganic substances are leached from submerged plants (Tukey 1970). For example, more than 85 % of C, N, and P leached from *S. alterniflora* was in organic form (Turner 1993). Carbohydrates, major components of the leaves themselves, are significant components of the leached organic matter (e.g. Opsahl and Benner 1999). In fact, ~34 % of the organic carbon loss during the leaching phase could be accounted for as *Spartina* carbohydrate carbon (Opsahl and Benner 1999). Leaching rates of organic components such as proline (released during salt stress) and other amino acids have been investigated (Pollak and Waisel 1970). Inorganic ions and compounds are also released during this phase (e.g. Bradley and

Morris 1991; Rozema et al. 1991). Tukey & Tukey (1958) found that more than 25 % of the sodium and manganese in young leaves could be leached in one day. They also found that 1-10 % of elements such as calcium, potassium, and magnesium were leached while less than 1 % of the chlorine, iron, phosphorus, and zinc were removed (Tukey and Tukey 1958). Release of other elements such as boron (Pulich 1978), sulfur (Pakulski and Kiene 1992), mercury, cadmium, zinc (Kraus et al. 1986), and selenium (Zawislanski et al. 2001) have also been studied.

C:N:P of leachate from Louisiana *S. alterniflora* averaged 164:10:1 compared to 794:46:1 for live plant tissues, which suggests that leachate is enriched in phosphorus relative to carbon and nitrogen (Turner 1993). Currin et al. (1995) found that $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values of standing dead *S. alterniflora* were distinct from isotopic values of live *S. alterniflora* while $\delta^{13}\text{C}$ did not change. These results indicate that carbon is removed from *S. alterniflora* leaves without fractionation, possibly abiotically, while nitrogen and sulfur containing components are decomposed through biological activity resulting in fractionation (Currin et al. 1995).

E. Leachate Fates

Many organisms can use the material leached from leaves (Penhale and Smith 1977; Thayer et al. 1978). For example, heterotrophic plankton are efficient consumers of the organics in leachates (Turner 1978; Wright and Coffin 1983). Meiofauna can also take up these leachates (Gallagher et al. 1976; Turner 1978). In fact, the rate of microbial respiration in marsh waters may be correlated with DOC leached from *S. alterniflora* (Gallagher et al. 1976; Wright and Coffin 1983). In addition, Findlay et al.

(1986) have confirmed high DOC to bacteria biomass conversion efficiencies (53 %) for aquatic bacteria growing on macrophyte leachates.

Thus, material leached from vascular plants during decomposition is integral to salt marsh functioning. As mentioned above, 5-10 % of the total net aboveground plant production of *S. alterniflora* in south Louisiana is released from plants through leaching (Turner 1976). Although these rates are low per unit biomass, total plant biomass is high (Smalley 1959; Turner 1993), and thus the contribution to coastal water metabolism is high. In addition, leached material appears to be converted directly and efficiently into bacterial biomass that is then available to filter and deposit feeders. Therefore, the contribution of leachates to the ecosystem is potentially significant.

III. Microbial Degradation: Second Phase of Decomposition

A. General Properties of Microbial Degradation

According to various authors (Benner et al. 1984a; Hodson et al. 1984; Valiela et al. 1985), after decaying plants undergo leaching, the second phase of decomposition is dominated by microbial activity. Compared to leaching, the rates of microbially mediated plant decomposition are slower (Saunders 1976) but last longer (Valiela et al. 1985) resulting in significant loss of organic matter (Newell and Fallon 1989; Newell et al. 1989; Haddad et al. 1992). Estimates of original plant material degraded during this phase range from 40 to 70 % (Valiela et al. 1985).

An interesting and widely observed trend found during microbially dominated decomposition of plant matter is that the percent of nitrogen in the litter increases over time (Godshalk and Wetzel 1978b; Rice and Tenore 1981; Valiela et al. 1985). This seems counterintuitive when considering nitrogen as a limiting nutrient, for one might

expect nitrogen to be preferentially removed from leaves. However, White (1994a) found that 50-65 % of the total nitrogen in detrital *S. alterniflora* was derived from external sources. The importance of external N sources is also supported by a study that found increased nitrogen pools in leaves during the spring/summer months coinciding with highest N₂-fixation rates (Newell 1992). The importance of this trend to microbial activity will be discussed elsewhere.

B. Factors Affecting Microbial Rates

Many factors affecting microbial decomposition rates of plant material have been identified and studied (e.g. Swift et al. 1979). Based upon their seminal study, Valiela et al. (1985) ranked factors controlling decomposition. In order of importance, they found species of litter, temperature, internal nitrogen content, external nitrogen content, and large detritus feeders to affect rates of microbially mediated plant decomposition.

Valiela et al. (1985) found the type of plant litter to be the most important factor determining rates of decomposition. Different litter types contain varying concentrations of lignocellulose and varying proportions of lignins, polysaccharides, and other plant material. This greatly affects plant degradability. For example, grasses have large proportions of cinnamyl phenols, ferulic acid, and *p*-coumaric acid (Sarkanen and Ludwig 1971) as monomeric components of lignin, which may increase decomposition by microbes (Hartley and Jones 1977).

Physical and environmental factors such as temperature, pH, salinity, submersion, and agitation may affect microbial decomposition of plant material as well. Higher rates of plant litter weight loss have been found at higher temperatures (White and Trapani 1982). In the environment, mass was lost at a slower rate during winter

months (Valiela et al. 1985). Another factor, pH, has been shown to influence solubilization of polysaccharides (Benner et al. 1985) and fungal lignolytic enzyme activity (Pointing et al. 1998) during vascular plant degradation. The effects of salinity on decomposition are mixed: the extent of fungal cellulolysis appears to be unaffected by changing salinities, while fungal peroxidase activity was favored by high salinities and fungal laccase activity was favored by lower salinities (Pointing et al. 1998). Although some studies suggest submersion time may be a factor in determining rates of microbially mediated decomposition (Kruczynski et al. 1978; White and Trapani 1982), Valiela et al. (1985) demonstrate that rates are not increased by time submerged. In addition, agitation has been proposed as a factor that may suppress fungal lignocellulolytic activity (Kirk and Farrell 1987; Eriksson et al. 1990).

Nitrogen content is also important in determining microbial decomposition rates of vascular plants. Higher decay rates are found with increased nitrogen supply (Haines and Hanson 1979; Marinucci et al. 1983). This is similar to the role played by nitrogen in decomposition of other types of organic matter (Mann 1976; Park 1976; Berg et al. 1982). Nitrogen supplied either internally (elevated nitrogen concentrations in the plant material) or externally (supplied as “atmospheric” nitrogen via N fixation) has the same effect on decay rates (Valiela et al. 1985). However, in natural settings, nitrogen content of leaves has only a slightly positive effect on decomposition rates (Valiela et al. 1985). This may indicate that not all leaf nitrogen is available to decomposers (Rice 1985). Because nitrogen has a positive effect on decomposition rates, the trend of increasing nitrogen content within decomposing leaves during the microbially-dominated stage of decomposition is important.

Although it received the lowest ranking of importance by Valiela et al. (1985), the presence of detritus-feeders and other animals can affect rates of plant decomposition during the microbially-dominated stage (Harrison and Mann 1975; Fenchel and Harrison 1976; Sherr et al. 1982). Some detritus-feeders such as *Littorina* snails have been shown to negatively impact decomposition during this phase (Valiela et al. 1985). This inhibition of microbial plant decomposition may be an indirect effect, but since *Littorina* effectively digest salt marsh-fungal mycelium (Newell 1993a), the plant decomposition rates may be directly impacted by the presence of detritus-feeders. Other organisms such as amphipods (Newell and Porter 2000), mussels (Newell and Barlocher 1993; Newell and Krambeck 1995; Newell and Porter 2000), and other invertebrates (e.g. McGonigle 1997) have been shown to ingest fungal and bacterial plant microbes and thus negatively impacting microbial decomposition. In contrast, some animals have been shown to enhance microbially mediated plant decomposition. For example, invertebrates such as marsh crabs and snails have been shown to shred the leaves, thus increasing surface area available for microbial degradation (Crichton 1960; Pfeiffer and Wiegert 1981; Bortolus and Iribarne 1999). Graça et al. (2000) found that invertebrates such as coffeebean snails (*Melampus bidentatus*) and amphipods (*Uhlorchestia spartinophila*), enhanced fungal growth on decaying *S. alterniflora* leaves.

C. Prokaryotes vs. Eukaryotes: Compartmentalized Decomposition

A study by Currin (1995) demonstrated that microbes preferentially degrade specific components of the lignocellulose fraction of plant material. $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values of live versus standing dead material in *S. alterniflora* changed, suggesting isotope fractionation was occurring. In particular, $\delta^{15}\text{N}$ values decreased as *S.*

alterniflora decayed suggesting selective remineralization of heavier nitrogen or input of nitrogen via nitrogen fixation. Even though plant N and S are impacted by microbial activity, $\delta^{13}\text{C}$ values did not change as *S. alterniflora* decayed. These results concur with others indicating that C-rich compounds like lignin are not selectively degraded while more N-rich compounds are preferentially utilized (Currin et al. 1995). Within the lignin fraction alone, evidence suggests preferential use of cinnamyl and syringyl phenols during *S. alterniflora* lignin degradation (Moran and Hodson 1990; Bergbauer and Newell 1992).

Both fungi and bacteria are equipped with enzymes to degrade lignocellulose, but each appears to have a separate niche for this process in a salt marsh.

Phaeosphaeria spartinicola, a leaf-inhabiting ascomycete fungus common to standing dead *S. alterniflora*, can degrade both the lignin and the polysaccharide portions of lignocellulose (Bergbauer and Newell 1992; Newell and Porter 2000). While most fungi and microbes possess the capability to degrade both portions of LC, fractions are used preferentially (Moran and Hodson 1990). Generally, fungi use less refractory compounds than bacteria when breaking down plant material. For example, Bergbauer and Newell (1992) found rates of polysaccharide decomposition by fungi to be high (23 % degraded after 45 days), while lignin oxidation rates are lower in fungal communities (2 - 3.3 % of lignin mineralized into CO_2 after 23 to 45 days of incubation) than in bacterial communities (Benner et al. 1984c; Benner et al. 1986; Bergbauer and Newell 1992). Even so, fungal degradation of lignin is far more efficient than that performed by bacteria, but many bacteria can use the catabolic intermediates from this process

(Ruttimann et al. 1991). In a study by Benner et al. (1986), bacteria were responsible for 75-85 % of LC degradation in saltmarsh samples.

D. Mechanisms of Microbially-Mediated Plant Degradation

Complete enzymatic hydrolysis of cellulose by marine fungi requires endoglucanases (E.C. 3.2.1.4), cellobiohydrolases (E.C. 3.2.1.91), β -glucosidase (E.C. 3.2.1.21), and possibly endo-type xylanases (Pointing et al. 1998). Endoglucanases catalyse the initial breakdown of cellulose to expose individual chains; cellobiohydrolases (E.C. 3.2.1.91) cleave cellulose into cellobiose units; while β -glucosidase hydrolyzes cellobiose to glucose (Eaton and Hale 1993). Hemicellulose degradation, less well understood, is thought to involve endo-type xylanase cleavage throughout the xylan polymer to yield xylobiosyl and xylotriosyl residues (Eaton and Hale 1993; Pointing et al. 1998).

The mineralization of lignin to CO_2 involves a mixture of enzymatic and non-enzymatic processes. In particular, three lignin modifying enzymes (LME's) are recognized as significant in lignin degradation by fungi (Boominathan and Reddy 1992). These include lignin peroxidase (E.C. 1.11.1.14), Mn-dependent peroxidase (E.C. 1.11.1.15) and laccase (E.C. 1.10.3.2) (Buswell 1991; Boominathan and Reddy 1992; Pointing et al. 1998). These LME's are non-specific and perform oxidation, hydroxylation, and cleavage of a variety of lignins and lignin-related compounds (Chen and Chang 1985).

IV. Bacteria and Fungi: Microbial Plant-Degraders

A. Spatial Distribution

In terrestrial ecosystems, fungi are generally considered to be the primary degraders of lignocellulose; but in aquatic systems and salt marsh sediments, bacteria appear to be the predominant degraders (Witkamp and Ausmus 1976; Kirk et al. 1977; Benner et al. 1984a; Benner et al. 1986; Newell and Fallon 1989; Newell et al. 1989; Haddad et al. 1992). On land, fungi control initial vascular plant decay (e.g. Boddy 1999; Kendrick 2000) partly because of their ability to penetrate leaf surfaces. This ability, however, declines with agitation (Kirk and Farrell 1987; Eriksson et al. 1990), suggesting that environments where physical agitation occurs, such as the water column, may favor bacterial over fungal hyphae growth (Newell 2002). In fact, rates of fungal-mediated decomposition are depressed in submerged conditions, especially when the aquatic environment is highly acidic (Benner et al. 1984c; Benner et al. 1985). A major advantage to bacteria in aquatic systems is their high substrate affinity. Low oxygen conditions in sediment give bacteria advantage over fungal decomposers there as well.

B. Temporal Distribution

Most studies of microbial decomposition find fungi to be initial degraders followed by bacteria (Kaushik and Hynes 1971; Iversen 1973; Mason 1976; Suberkropp and Klug 1976; Lee et al. 1980; Federle and Vestal 1982); however, a few studies found the opposite trend (Morrison et al. 1977; Newell 1981; Rublee and Roman 1982). Suggested mechanisms for this sequence include inhibition of bacteria by fungi during the initial processing of the leaves (Chamier et al. 1984) and better use of soluble

compounds (derived through leaching, decomposition, or both) by bacteria in later stages (Suberkropp and Klug 1976). While biological interactions may play a role in determining the dominance of these two groups, physical and chemical factors appear to be more important determinants of dominance.

A single *Spartina* shoot can have live leaves, standing dead leaves, and dead leaves collapsed onto the sediment. Each of these stages creates subtly different habitats for microbial communities. In standing dead *S. alterniflora* leaves, for example, fungi dominate the microbial community (Newell and Fallon 1989). However, when dead *S. alterniflora* leaves submerge or collapse onto the sediment, fungi become relatively rare (Newell et al. 1989). In the sediments, bacteria are responsible for mineralizing most of the *S. alterniflora* lignin and polysaccharides.

The co-presence of fungal and bacterial decomposers has been studied via direct counts (e.g. Newell and Hicks 1982), production rates (e.g. Newell et al. 1989), culturing, and molecular techniques (Buchan et al. 2003). Regardless of which group is dominant, metabolically active fungi and bacteria co-occur on vascular plant detritus for much of the decomposition process (e.g. Gessner and Kohlmeyer 1976; Gessner 1977; Newell and Hicks 1982; Buchan et al. 2002).

C. Interactions Between Bacteria and Fungi

Fungi and bacteria may form mixed consortia that interact in complex ways to accelerate or retard decomposition (Newell and Porter 2000). Biotic interactions such as antagonism, synergism, competition, or even predation are possible between these two groups of organisms. Abiotic parameters may also determine relative contribution of fungi and bacteria (Newell 1996; Newell and Palm 1998). In addition, processes

such as bacterial nitrogen fixation and fungal substrate pervasion lead to variation in decomposition abilities for these groups. These biotic interactions, environmental variables, and varying decomposition abilities all outline possible interaction mechanisms between fungal and bacterial plant degraders.

An increasing number of studies have focused on the interaction between fungal and bacterial plant decomposers. Beech leaf decomposition in streams is one common experimental system used to address this topic. For example, Bengtsson (1992) performed microcosm studies in this system and found a synergistic relationship between fungi and bacteria in which each group grew faster in the presence of the other. Further, Bengtsson found that bacteria had a more beneficial effect on fungal growth than fungi had on the bacteria (Bengtsson 1992). However, in a different microcosm study using beech leaves, Møller et al. (1999) found an antagonistic relationship between fungi and bacteria.

A recent study by Mille-Lindblom and Tranvik (2003) addressed the question of fungal and bacterial interaction using decomposing *Phragmites* culms in microcosm experiments. By inoculating sterilized *Phragmites* culms with various combinations of bacteria and fungi, they were able to show an antagonism between bacteria and fungi, similar to the results of Møller et al. (1999). However, even though the antagonism resulted in reduced biomass of each group, overall differences in community respiration between the various treatments were minor.

D. Fungal Decomposers of *Spartina*

In salt marsh systems, ascomycetes are the predominant fungi and are capable of degrading every component of a *Spartina alterniflora* plant (Kohlmeyer and

Volkman-Kohlmeyer 1991; Newell et al. 1996; Fell and Newell 1998; Kohlmeyer et al. 1999). In addition, these ascomyetes make up distinctly different fungal communities depending upon their location on the *Spartina* plant (Gessner 1977; Newell 1996).

Several methods have been used to identify plant-associated fungi. The most traditional methods are those of direct identification. Ascospore taxonomy and direct microscopy of leaf surfaces are commonly employed to identify fungi based upon morphology. Other traditional methods combine culturing with direct identification. Ascospore-drop procedures, conidia micromanipulation, and ascospore nichrome-needle micromanipulation are all culture dependent identification techniques. Recently, however, new molecular tools have been developed to identify fungi. For example, Buchan et al. (2002) used terminal restriction fragment length polymorphism (TRFLP) analysis of fungal internal transcribed spacer (ITS) regions of rRNA genes to describe the ascomycete population living within senescent *Spartina alterniflora* leaves. This technique complements results found by more traditional methods; however, molecular techniques generally provide for more rapid sample processing for fungal community structure after methods are optimized.

All approaches indicate the presence of ascomycetes inhabiting senescent *S. alterniflora* leaves. The two most common species, *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. 2, are often found in association with each other (Gessner 1977; Kohlmeyer and Kohlmeyer 1979; Leuchtmann and Newell 1991; Bergbauer and Newell 1992; Newell et al. 1996; Newell et al. 2000; Newell and Porter 2000; Newell 2001a; Buchan et al. 2002). Morphologically, the four-chambered ascospores of *P. spartinicola* are about 2-5 times larger than those of *Mycosphaerella* sp. 2. Tan to yellow aerial

mycelium of *P. spartinicola* is abundant in cultures while aerial mycelium of *Mycosphaerella* sp. 2 is less abundant and gray to tan in color (Kohlmeyer and Kohlmeyer 1979; Leuchtman and Newell 1991). Other common fungal species in this system include *Phaeosphaeria halima*, *Buergenerula spartinae*, and an unidentified ascomycete called '4cft' (Buchan et al. 2002).

The amount of fungi present can be measured in several ways. Typical methods for direct counting include harsh treatments to separate the fungi from the leaf substrate. If leaves are homogenized, the resulting solution can be gelled with agar and dried or stained with a fluorescent dye or filtered onto a membrane; these products can then be examined microscopically to assess fungal biovolume (Jones and Mollison 1948; Hannsen et al. 1974; Paul and Johnson 1977; Frankland et al. 1978). As an alternative to homogenization, the litter can be cleared chemically before a stain is applied and fungal hyphae can be enumerated microscopically (e.g. Barlocher and Kendrick 1974). Indirect methods for fungal quantification have also been developed. Immunoassays and glucosamine: muramic acid ratios have been used as indirect biochemical assays for fungal quantification. A more powerful tool, however, is to measure ergosterol since it is the primary membrane sterol in almost all ascomycetes, it is lacking in plants, and the few microbes found to synthesize ergosterol are not embedded in senescent vascular tissue (Lee et al. 1980; Newell 1992; Newell 2000; Gessner and Newell 2002).

Ergosterol content of leaves is a relative measure of living fungal biomass because the fungal membranes containing ergosterol are quickly lysed upon hyphal death (Ekbald et al. 1998; Newell 2000; Gessner and Newell 2002). Even though living

fungal biomass in standing dead *S. alterniflora* can make up 10-20 % of the plant organic matter (Newell 1993a), dead fungal biomass may equal live fungal biomass (Newell 1996).

E. Bacterial Decomposers of *Spartina*

Whereas fungal diversity on *Spartina alterniflora* has been categorized extensively, data on bacterial diversity in this system are rare. Most of the published data either focused on diazotrophs or sediment-inhabiting bacteria (e.g. Bagwell et al. 1998; Lovell et al. 2001; Burke et al. 2002; Brown et al. 2003). However, recent molecular-based studies have taken steps to characterize the general bacterial consortium inhabiting and degrading aboveground leaves of *Spartina alterniflora* (Buchan et al. 2003).

Microbiologists have been finding ways to classify bacteria for many years, which is reflected in the various methods for their identification. Traditional procedures rely on culture techniques so that the external morphology, Gram-stain classification, nutritional requirements, biochemical properties, or surface proteins can aid in overall bacteria identification. With the aid of molecular techniques, the need to rely on culturing is reduced. For example, community DNA can be extracted from a particular habitat, and eubacterial primers can be used in a polymerase chain reaction (PCR). The products can then be subjected to TRFLP analysis or a functionally similar process called denaturing gradient gel electrophoresis (DGGE) to get a fingerprint of the bacterial community members. When using DGGE, bands can be excised from the gel for further sequencing analysis. Direct sequencing of bands or clones is a more definitive

and precise method for identifying bacteria in this *Spartina* environment than traditional culture-based methodology.

Several studies have established that LC-degrading (Moran and Hodson 1990) and lignin-degrading bacterial consortia (Gonzalez et al. 1996) are present in Georgia's salt marshes. A recent study by Buchan et al. (2003) used a combination of clone libraries, culture collections, and TRFLP analyses to elucidate the bacterial community present in the *Spartina* decay system on Sapelo Island, Georgia. Their results indicate that the vast majority of bacteria present were α -Proteobacteria. This agrees with González and Moran (1997) who found that up to 28 % of bacterial DNA in Georgia's salt marsh water belongs to the marine class of α -Proteobacteria. According to Buchan et al. (2003), next in abundance when using non-culture based methodology were members from the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group followed by γ -Proteobacteria. When using culture techniques, Gram positive bacteria are also found in this system (Buchan et al. 2003).

The amount of bacteria present can be measured in several ways. The typical method for direct counting includes staining the bacterial DNA/RNA with dyes such as acridine orange (AO) or 4',6-diamidino-2-phenylindole (DAPI) before microscopically enumerating cells (Hobbie et al. 1977; Porter and Feig 1980). This technique, although reliable, is time-intensive and relatively subjective. A faster approach to counting bacteria cells is to use the process of flow cytometry (Joachimsthal et al. 2003). After staining cellular DNA/RNA, bacterial cells can be counted by passing a thin stream of aqueous sample material through a laser beam. Depending on the incidence and scatter angles of the laser beam, bacteria can be enumerated by comparison with an

internal bead standard of known concentration. Although this technique for counting bacteria is not the most common, several studies using monocultures or environmental aquatic samples have documented that flow cytometric counts are comparable to epifluorescence counts (Monfort and Baleux 1992; Joachimsthal et al. 2003).

An important factor in accurately counting bacteria by flow cytometry is the choice of stain used. Several dyes such as Hoechst dye, DAPI, TOTO, and SYTO 13 have been used with varying accuracy (Monfort and Baleux 1992; Monger and Landry 1993; Li et al. 1995; delGiorgio et al. 1996; Marie et al. 1997; Andreatta et al. 2001; Li and Dickie 2001). In a recent study by Lebaron et al. (1998), a particular class of dyes (blue nucleic acid dyes from Molecular Probes, Inc.) was tested in various aquatic sample types. They found that using SYBR Green II for fixed or unfixed seawater samples decreased background noise while increasing accuracy in measurement (Lebaron et al. 1998).

V. This Study

The purpose of this current study was to examine the interaction between ascomycetes and bacteria in the initial *Spartina* decay system by asking three main questions. First, how does the presence of ascomycetes modify decaying *Spartina* leaves and leachate? Second, how are bacteria affected by fungally-modified leachate added as a growth substrate? Third, could bacterial chitinase activity play a role in interactions between fungi and bacteria in the *Spartina* decay system? These questions were addressed by physically separating bacteria from fungi in two-stage microcosm experiments in an attempt to better characterize possible interactions within the *Spartina* decomposer consortium.

CHAPTER 2

METHODS

I. Site Description and Environmental Sample Collection

All environmental samples were collected from Dean Creek marsh on Sapelo Island, Georgia (Figure 2.1; 31°23' N; 81°17' W: (Pomeroy and Wiegert 1981)), Site 6 of the Georgia Coastal Ecosystems Long Term Ecological Research program (GCE-LTER; <http://gce-lter.marsci.uga.edu/lter/>) as well as a site for the Sapelo Island Microbial Observatory (SIMO; <http://simo.marsci.uga.edu/>). Semidiurnally flooded, this marsh is dominated by *Spartina alterniflora*, a cordgrass commonly found along the western Atlantic coastline (Dardeau et al. 1992; Dai and Wiegert 1996; Bertness and Pennings 2000; Mendelssohn and Morris 2000). Dean Creek itself is a tidal channel characterized by water depths of 1-2 m and daily tides of up to 3 m.

Leaf and water samples were collected in November, 2001 and May, 2002 from Dean Creek marsh to use in the experiments described below. Each time, early decay (standing dead) leaves of *Spartina alterniflora* were clipped and stored in plastic bags at 4° C until further use. All water used in the experiments was collected at high tide from Dean Creek and stored in carboys washed with 10 % HCl.

II. Experimental Design

In order to study how fungal degradation affects bacterial growth and communities, microcosms were set up in two stages: one of fungal growth and one of

bacterial growth. This was designed to physically separate the fungal and bacterial components of the *Spartina* degradation system. Because of this separation, the presence, absence, and type of fungi could be controlled in different treatments. Thus, bacterial response to different leaf/fungal leachates could be monitored.

A. Fungal Stage

The top chamber of the microcosm included dead *Spartina alterniflora* leaves and the fungi inhabiting them. Standing dead *Spartina* leaves were sterilized, partitioned into treatments, and inoculated as described in Table 2.1. Three control treatments were created: Empty, Control, and Natural. The Empty control contained no leaves and no fungi; this was used to observe the effect of microcosm container and airflow system on the experimental outcome. The Control treatment held only autoclaved leaves but was not inoculated with fungi; this treatment controlled for abiotic leaching from the leaves. In the Natural treatment, leaves that had not been sterilized were used without further fungal inoculation; this positive control held the natural population of fungi, bacteria, protists, and in some cases, insects or snails.

The two fungi used to inoculate experimental treatments were chosen to reflect dominant ascomycete fungi from Sapelo Island's salt marshes. The three treatments were autoclaved leaves inoculated with *Phaeosphaeria spartinicola* (PS), *Mycosphaerella* sp. 2 (MS), or a combination of both *P. spartinicola* and *Mycosphaerella* sp. 2 (PS+MS).

The microcosms, performed in duplicate, were maintained for 39-51 days in an air-flow system that met three main growth conditions of fungi: access to light, air (nitrogen and oxygen), and moisture. This system was comprised of eight "stations"

(Figure 2.2); each system could accommodate three microcosms. All glassware, plastic couplings, Swinnex filters, and filter chambers were acid washed in 10 % HCl. The Milliflex tubing, in-line filters, and assembled Sterifil® chambers were then wrapped in aluminum foil and autoclaved (121° C, 30 minutes) before use. Glassware was ashed as well (450° C, 5 hours). Microcosms were flushed with sterile, scrubbed, humid air (Figure 2.2).

Every four days, autoclaved Dean Creek water was added to the Fungal Stage to moisten leaves and rinse them of any degradation intermediates, leachate, or other soluble and microparticulate plant/fungal products. This rinse (leachate) was collected and added to the Bacterial Stage (Figure 2.3).

B. Bacterial Stage

Filtered Dean Creek water (1 µm polycarbonate, Nuclepore) served as the original inoculum. Each time the microcosms were rinsed, 10 mL of that leachate was added to each bacterial enrichment culture as outlined in Table 2.2. An unmanipulated portion containing both particulate and dissolved portions of leachate was added to the first treatment (P+D). The remaining leachate was filtered to remove particulates and added to the second and third bacterial treatments. Only dissolved material was added to the second treatment (D). Suspended chitin was added to the third bacterial treatment (D+C) in addition to dissolved material to compensate for hypothesized loss of particulate chitin due to filtration. Duplicate enrichment cultures were grown in 300 mL Erlenmeyer flasks in the dark at ambient temperature (23°-25°C) for 24-44 days and shaken at least once a day during that period.

III. Experimental Procedure

A. Inoculation

1. Leaves

Leaves were first rinsed with cold deionized water (DI water) to remove clay (Newell 2001b) before the tips were discarded. Pieces 4 cm long were cut from within 12 cm of the ligule end of the leaves and pooled. Depending on treatment, some leaves were then sterilized by autoclaving (121° C, 30 minutes). Prior to these experiments, gamma irradiation was tested as a sterilization method. *Spartina* leaves were rinsed with tap water, cut into 4 cm lengths, and exposed to varying levels of gamma irradiation (⁶⁰Co). Following exposure levels ranging between 0 and 300,000 rads, several leaves were placed on solid V8 agar plates and incubated under natural light and temperature conditions. After just 2 days of incubation, each agar plate had visible fungal growth. Even though the fungi were not identified, it is possible that some ascomycetes in the *Spartina* decomposition system are resistant to sterilization methods.

Subsamples of leaves were collected for analyses described in greater detail below. Each microcosm received 25 g wet weight of leaves. For Experiment I, these leaves were autoclaved, placed into the microcosms then immediately inoculated with fungi. For Experiment II, the autoclaved leaves were placed inside of the microcosms, and the assembled microcosms were autoclaved again. These leaves were observed for two weeks to detect possible fungal contamination (Table 2.3), and then inoculated with fungi.

Two fungi were used for the fungal treatments: *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. 2. Pure cultures of isolates were obtained from J. I. Lyons (UGA Marine Science Department; *P. spartinicola* = SAP 132; *Mycosphaerella* sp. 2 = SAP 133). Cultures were maintained either on nutrient rich agar (1 g ground *Spartina* leaves, 0.4 g malt, 0.4 g yeast extract, 0.2 g NaNO₃, 0.3 g K₂HPO₄, 1.4 g KH₂PO₄, 16 g agar, volume up to 1 L with 20 ppt sea water) or low nutrient V8 media (2 % V8 [v/v; Campbell Soup, Inc.], 2 % agar [w/v], 15 ppt sea water) at room temperature in indirect sunlight.

Leaves were inoculated in a laminar flow hood using sterile inoculation loops. For the PS and MS treatments, two plugs of fungal hyphae grown on low nutrient V8 agar were added to the autoclaved leaves. One plug of each type of fungi was added to the PS+MS treatment. The control treatments (Empty, Natural, Control) did not require fungal inoculation, but to compensate for any nutrient addition, two plugs of sterile low nutrient V8 agar were added per microcosm. Approximate volume of plugs ranged from ~81 μ L and ~125 μ L. Representative plugs were added to 10 mL of ethanol, stored in the dark at 4^o C, and later analyzed for ergosterol content.

After inoculation, the microcosms were completely closed, sealed with parafilm, and connected to the airflow system. Up to three microcosms were maintained per airflow system, and these microcosms were replicates/triplicates of a particular treatment. Inoculated microcosms were not disturbed for one week prior to the first “rinse.”

2. Water

Dean Creek water was used in two capacities for this experiment: as bacterial inoculum and as “rinse” water added to the Fungal Stage to rinse the leaves of any

degradation intermediates, leachate, or other plant/fungal products. As such, each required different filtration regimes.

a. Bacterial Inoculum

Dean Creek water used for bacterial culture inoculum was 1.0 μm filtered (Poretics, polycarbonate) by slight vacuum and checked microscopically for protists. Two hundred mL of this water was transferred to each 300 mL Erlenmeyer flask used for the Bacterial Stage. All flasks were plugged with cheese-cloth-wrapped cotton and incubated in the dark at room temperature for the duration of the experiment.

b. "Rinse" Water

Filtered Dean Creek water served as the "rinse" water added to the microcosms. It was first filtered through ashed GF/F filters (particle retention $\sim 0.7 \mu\text{m}$) before final filtration through either Sterivex filters (Millipore, 0.22 μm pores) or 0.2 μm polycarbonate membranes (Poretics). This water was then autoclaved in 500 mL portions (121° C, 30 minutes) and stored in glass containers at room temperature.

B. Collection and Storage

The sampling regime can be broken down into three activities: rinse days, Bacterial Stage days, and beginning/midpoint/endpoint sampling days. The first sampling day was considered Day 0. Rinse days occurred every 4 days starting on Day 0 while Bacterial Stage sampling occurred every 4 days starting on Day 2. This pattern proceeded for the duration of each experiment. Midpoint and endpoint sampling days are given in Table 2.3. A description of sampling procedure is outlined below. All subsampling took place in a laminar flow hood unless otherwise noted.

1. *Rinse Days*

On Rinse Days, 90 mL of 0.22 μm filtered and autoclaved Dean Creek water was sprayed over the leaves. The water was injected through a rubber-stoppered port in the microcosm lid by attaching a 20-gauge needle to a 60 mL syringe. It took between 5-10 minutes to add the water to each microcosm, making sure water was sprinkled throughout the upper chamber. The water was then allowed to drain into the lower chamber of the microcosm for at least 30 minutes; this rinse water will now be referred to as leachate. To collect this leachate, the rubber cap on the lower chamber port was replaced with a 60 mL syringe. The liquid was swirled before collection in the syringe, and the final volume collected was recorded.

Ten mL of leachate was added to Particulate + Dissolved (P+D) bacterial treatment replicates. The remainder was filtered through a pre-weighed 25 mm diameter ashed GF/F filter. Ten mL of this filtered leachate was added to each of the Dissolved (D) and Dissolved + Chitin (D+C) bacterial treatment replicates. Any remaining filtered leachate was collected in 10 % acid-washed vials (Nalgene® HDPE) and stored frozen at -20°C until analysis. The total volume of leachate passing through the GF/F filter was recorded, and the filter was stored frozen at -20°C until analysis. Next, 10 μL of 14 ppt (w/v) chitin suspension (Sigma, crabshell), was added to each D+C bacterial treatment replicate. Each culture was then gently swirled to mix the enrichment material into solution.

Last, subsamples of the bacterial cultures (2-8 mL) were collected for bacterial counts. The volume removed was chosen to keep the total culture volume as constant

as possible. Samples were preserved with borate-buffered formalin (Sigma; 6 %, v/v) then stored at 4° C until analysis.

In between rinse days, the lower chamber of each microcosm was rinsed with 3 washes of autoclaved Milli-Q water. This water was added via a dedicated 60 mL syringe after removing the rubber cap from the lower chamber port. After swirling the water, it was removed through the same port by a separate syringe and discarded.

2. *Bacterial Stage Days*

Two days after each “rinse” day, the bacteria cultures were sampled again. Two mL samples were collected and stored for enumeration as described above.

3. *Beginning/Midpoint/Final Days*

Each treatment was sampled for extensive analysis three times during each experiment: initial, midpoint, and endpoint (Table 2.3). At least one fungal microcosm per treatment was sacrificed at midpoint and endpoint to collect the leaves. Two cm portions of 3 leaves were added to 10 mL of ethanol and stored in the dark at 4° C for subsequent ergosterol analysis. Ten leaves were randomly selected and stored in aluminum foil at -20° C until DNA was extracted. Another 15 leaves were collected for organic density analyses and dried (60° C overnight), while the remaining leaves were used for CHN analysis.

Bacterial cultures were subsampled at midpoint then sacrificed at the end. During subsampling, 5 mL of culture were placed in 20 mL scintillation vials and incubated with artificial substrate to assay chitinase activity as described below. At midpoint, 50 mL of each culture was filtered through a Sterivex (Millipore, 0.22 µm pores) and the filter was stored in 1.8 mL lysis buffer (0.75 M sucrose, 40 mM EDTA, 50

mM Tris [pH 8.3]) at -20°C until DNA extraction. At the endpoint sampling, the remaining culture was filtered and stored in the same manner.

IV. Analytical Procedures

A. Leaves

1. Ergosterol Content

Spartina pieces and V8 culture plugs were sent to S. Y. Newell (University of Georgia, Marine Institute) for ergosterol analysis. As previously described (Newell 1993b; Newell 2000), samples were sequentially extracted in methanol then pentane and taken through high-performance liquid chromatography (HPLC) along with procedural standards of pure ergosterol. Values were adjusted to reflect the amount of ergosterol per unit of leaf ash free dry weight.

2. DNA Extraction, PCR, TRFLP

DNA was extracted from the *S. alterniflora* leaves and purified using Mega Size Soil DNA Extraction Kits (MoBio, Solana Beach, CA). Ten leaf blades from each microcosm were extracted per kit following manufacturer instructions.

Extracted DNA was used as template for ascomycete-specific ITS region PCR amplification. The primers used, ITS 1F and ITS4A (Table 2.4), amplify a ~600bp product that includes the ITS 1, 5.8S, and ITS2 regions of the rRNA operon (Larena et al. 1999; Buchan et al. 2002). PCR was carried out by using Ready-To-Go PCR Beads (Amersham Pharmacia, Piscataway, NJ) with $0.2\ \mu\text{M}$ concentration of each primer and 50 ng of DNA using a DNA Engine thermocycler (MJ Research), with an initial denaturation for 3 minutes at 95°C followed by 35 cycles of 1 minute at 95°C , 30

seconds at 52° C, and 1 minute at 72° C. A final extension step of 10 minutes at 72° C was included to complete any partial polymerizations.

Products were recovered from a 1 % (w/v) agarose gel with the QiaSpin Gel Extraction Kit (Qiagen). The amplicons were then digested in a 20 µL reaction containing 100 ng of purified PCR product and 10 U of the restriction enzyme *HaeIII* (Roche, Indianapolis, IN) for 3 hours at 37° C. Digested samples were precipitated overnight (80 µL DI water, 10 µL 7.5 M ammonium acetate, 1 µL 0.07 % glycogen, 300 µL cold ethanol). The resulting pellet was resuspended in 12 µL of deionized formamide with 1 µL of DNA fragment length standard GeneScan-2500 (TAMRA; Applied Biosystems) and denatured (95° C, 5 minutes). The terminal restriction fragment lengths were determined on an ABI PRISM 310 in GeneScan mode (Buchan et al. 2002).

3. *Organic Density, Percent Ash*

Leaves were rinsed, measured for leaf area, then dried in an oven at 37-55° C for 2-10 days. Each leaf was weighed to obtain dry weight. Leaves were then ashed in a muffle oven at 450° C for 5 hours and re-weighed.

To calculate organic density, the weight of organic material (ash-free dry weight, AFDW) was divided by the leaf area. Percent ash was calculated using the following equation:

$$\text{Percent Ash} = \frac{\text{Dry Weight} - \text{Organic Material Weight}}{\text{Dry Weight}} \times 100$$

4. *Carbon and Nitrogen*

The remaining leaves were dried in an oven at 37-55 ° C for > 2 days then ground using a Wiley Mill (size 40 mesh). Ground samples were weighed on a microbalance (CAHN C-35) and analyzed for total carbon and nitrogen using a FlashEA™ 1112 Elemental Analyzer (ThermoQuest, Italia S.p.A). L-aspartic acid (ThermoQuest) was used as a standard.

B. GF/F Filters

Filters were analyzed to characterize the particulate component of leachate. Both the weight and carbon:nitrogen composition of the particles was determined in this analysis.

1. *Particles*

GF/F filters were dried in an oven (37-55° C) for 2-10 days before being weighed. In order to correct for different volumes of solution filtered, this equation was used:

$$Final\ Weight\ (mg) - Initial\ Weight\ (mg) \times \frac{1000\ mL}{Volume\ Filtered\ (mL)} = mg / L\ Particles$$

Since the volume of leachate that passed through each filter was known, the total weight of leached particles was calculated as well. In order to correct for background particles, weights collected in the Empty treatment were subtracted from other treatments.

2. *Carbon and Nitrogen*

GF/F filters were dried in an oven (37-55° C) for 2-10 days. They were then analyzed for total carbon and nitrogen using a FlashEA™ 1112 Elemental Analyzer (ThermoQuest, Italia S.p.A). L-aspartic acid (ThermoQuest) was used as a standard.

C. Dissolved Leachate

Dissolved organic carbon, total nitrogen, total phosphorus, and total carbohydrates were measured in leachate samples.

1. *Dissolved Organic Carbon (DOC)*

Two mL of leachate was acidified in ashed glass tubes to a pH ~3 using concentrated phosphoric acid. DOC concentrations were determined by high temperature catalytic oxidation (Sugimura and Suzuki 1988) using a TOC-5000 (Shimadzu) with three overlapping, 4-point potassium biphthalate calibration curves.

2. *Total Dissolved Nitrogen (TN) and Phosphorus (TP)*

TN and TP were determined by the persulfate oxidation method of Valderrama (1981). Five mL of sample was placed in ashed 20 x 125 mm Pyrex test tubes, then 0.7 mL of persulfate oxidation reagent (50.0 g potassium peroxydisulfate, 30.0 g boric acid, 350 mL 1 M NaOH, up to 1 liter volume with Milli-Q water) was added. Tubes were sealed with Teflon lined caps and autoclaved for 30 minutes at 121° C.

For TN measurements, duplicate aliquots of the oxidized solution were neutralized to a pH between 7-8 using 0.1 N HCl before being reduced via spongy cadmium incubation (Jones 1984). Briefly, 0.2 mL of ammonium chloride solution (0.7 M ammonium chloride; pH 8.5 with NaOH) was added to 1 mL samples. Spongy cadmium (0.7-0.9 g) was then added to each sample and incubated at room temperature for 60-90 minutes with gentle shaking. Then, 50 µL of a colorizing reagent

(50 mL phosphoric acid, 400 mL water, 5 g sulfanilamide, 0.5 g N-1-naphthylethylenediamine dihydrochloride, volume to 500 mL with water) was added to 1 mL of the sample. After 15 minutes of color development, absorbance at 540 nm was measured on a spectrophotometer (Shimadzu UV160U). Total dissolved nitrogen was calculated from a standard curve prepared with KNO_3 .

Orthophosphate was determined in duplicate subsamples of the oxidized solution using the method of Murphy and Riley (1962). Briefly, 0.1 mL of a colorizing reagent (100 mL ammonium molybdate solution [15 g ammonium paramolybdate, 500 mL DI water], 250 mL sulfuric acid solution [140 mL sulfuric acid, 900 mL DI water], 100 mL ascorbic acid solution [27 g ascorbic acid, 500 mL DI water], and 50 mL potassium antimonyl-tartrate solution [0.34 g potassium antimonyl-tartrate, 250 mL DI water]) was added to 1 mL of sample. After 15 minutes of color development, absorbance at 885 nm was quantified on the spectrophotometer (Shimadzu UV160U). Total dissolved phosphorus was determined using a standard curve prepared with KH_2PO_4 .

3. *Total Carbohydrates*

Selected samples were sent to the University of Georgia's Complex Carbohydrate Research Center for total glycosyl composition analysis using methodology previously described (York et al. 1985; Merkle and Poppe 1994). Briefly, aliquots of sample were freeze-dried and methylated by reaction with 1 M HCl and methanol at 80° C for 18-22 hours. The resulting methyl glycosides were *N*-acetylated with pyridine and acetic anhydride in methanol to detect amino sugars. After per-*O*-trimethylsilylation (Tri-Sil [Pearce]; 80° C; 30 minutes), samples were analyzed using

gas chromatography/mass spectrometry (GC/MS; HP 5890 GC interfaced to a 5970 mass selective detector using a Supelco 2330 fused silica capillary column).

D. Bacterial Cultures

1. Bacterial Counts

Bacteria were counted using flow cytometry (Lebaron et al. 1998). Fifty μL of a sonicated suspension of Fluoresbrite™ Carboxylate YG 1.0 μm Microsphere beads ($\sim 10^7$ beads/mL; Polysciences, Inc.) was added to 1 mL of fixed culture sample. SYBR® Green II RNA gel stain (Molecular Probes; 1 X final concentration) was then added to the samples which were incubated in the dark for at least 10 minutes before analysis using a FACSCalibur flow cytometer (Becton Dickson).

The resulting counts were then analyzed using CELLQuest software (Becton Dickson). Bacterial cells were distinguished from the beads by plotting side scatter against fluorescence. On the same day as flow cytometric measurements, 50 μL of the bead solution was enumerated microscopically (Leica DMRXA) on a 0.22 μm black polycarbonate filter (Poretics).

These data were then used to calculate the bacterial abundance (BA):

$$BA = \frac{\text{Beads}}{\text{mL}} \times \frac{\text{Bacteria}}{\text{Beads}} \times \frac{50 \mu\text{L}}{1100 \mu\text{L}}$$

$\frac{(\text{slide count})}{\text{mL}} \times \frac{(\text{flow count})}{(\text{flow count})} \times \frac{\text{bead volume}}{\text{total volume}}$

Selected bacteria samples were also enumerated by direct microscopic counts. After staining with DAPI (4', 6-diamidino-2-phenylindole; Sigma) for 5 min in the dark, samples were filtered onto a 0.22 μm black polycarbonate filter (Poretics), and bacteria were counted microscopically (Leica DMRXA). These samples were also examined to determine the presence of protists in cultures.

2. *DNA Extraction, PCR, DGGE, Sequencing*

Total community DNA was extracted from the samples as previously described (Ferrari and Hollibaugh 1999; Bano and Hollibaugh 2000). Samples in Sterivex cartridges were allowed to thaw before adding 40 μL of lysozyme (50 mg/mL) to each. They were then incubated for 60 minutes at 37° C. Then 50 μL of proteinase K (20 mg/mL) and 100 μL of 20 % (w/v) sodium dodecyl sulfate was added to each cartridge and incubated at 55° C for 2 hours. The lysate was removed and placed into fresh 2 mL microcentrifuge tubes. Eight hundred μL of lysate was purified by sequential extraction with 800 μL phenol-chloroform-isoamyl alcohol (25:24:1; Ambion), chloroform-isoamyl alcohol (24:1; Sigma), and finally *n*-butanol (Sigma). The final aqueous phase was removed, placed in a Centricon-100 concentrator (Amicon), mixed with 500 μL of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), and centrifuged at 1,000 X *g* for 10 minutes. Five hundred μL of TE was added to the Centricon and centrifuged for another 10 minutes. Successful extraction of high molecular weight DNA was assessed by electrophoresis of the sample on a 1 % agarose gel; extracted DNA was then stored at -20° C until amplification.

The variable 3 region of the 16S rRNA gene was amplified by the polymerase chain reaction using 17 bp primers complementary to positions 341-358 (341f) and 517-

534 (534r) of the *E. coli* 16S rRNA gene (Table 2.4). A 40-bp GC clamp was added to the 5' end of the 341f primer. PCR was carried out two ways. The first way used PCR beads as outlined above. In the second method, PCR reaction mixtures were prepared in a total volume of 25-100 μ L and contained 1 X PCR buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50 % glycerol, and 1 % Triton X-100 [pH 8.0]), 2.5 mM MgCl₂, a 200 μ M concentration of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), a 0.5 μ M concentration of each primer, and 10 to 100 ng of template DNA.

A touchdown PCR protocol was used (Don et al. 1991) with a hot start (Erlich et al. 1991) similar to previous methods (Ferrari and Hollibaugh 1999; Bano and Hollibaugh 2000). A DNA Engine thermocycler (MJ Research) was used with the following program. Hot start denaturation occurred at 94° C for 5 minutes with a pause at 82° C to add *Taq* polymerase (2.5 U; Promega). Initial denaturation occurred at 94° C for 3 minutes with subsequent denaturation for 30 sec.; annealing for 30 sec. at 65-55° C (stepping down 1 degree per cycle from 65 to 62° C and 1 degree every other cycle from 62 to 55° C); and extension at 72° C for 30 seconds. This was followed by 25 cycles of 30 second denaturing (94° C), annealing (55° C), and extension (72° C) with a 10 minute extension on the final cycle. Successful amplification was assessed by electrophoresis of the sample on a 1 % agarose gel.

PCR products were concentrated by ethanol precipitation using 2 x volume of ice-cold ethanol and 0.1 x volume of 3 M sodium acetate (pH 5.2) overnight at -20° C. This was then centrifuged at 12,000 x g for 30 minutes at 4° C. The DNA pellet was

dried in a DNA SpeedVac (Savant) for 10 minutes and resuspended in 15 μ L Milli-Q water or TE buffer (pH 8.0).

These PCR products were then used to perform denaturing gradient gel electrophoresis (DGGE) analysis (Ferrari and Hollibaugh 1999). For each sample, 200-500 ng of PCR product was loaded on a 6.5 % polyacrylamide gel with a 45-65 % denaturing gradient. Gels were run for 10 hours in 1 x TAE buffer (National Diagnostics) at a constant voltage (75 V) and temperature (60° C). Gels were scanned using a FMBIO II (Hitachi) gel scanner set to measure fluorescein fluorescence.

Resulting images were analyzed using Molecular Analyst Fingerprint Plus® software (BioRad) in a process similar to Ferrari and Hollibaugh (1999). Gel images were first digitized, lanes were defined, and then bands were normalized to lane standards. For this data set, lane standards consisted of a mixture from six clone/standard PCR products in equal molar concentrations (Table 2.5). After normalization, gels were compared for band-based similarity by manually editing automatic band calls (heights \geq 1 % above the local background and areas \geq 0.5 % of the total area under the densitometric curve). Tolerance for band position was set at 0.7 % of the total curve length to provide similarity dendrograms. Banding pattern similarities were calculated using an UPGMA clustering algorithm and the Jaccard band similarity coefficient. Using the same tolerance settings, band positions were numbered to aid in comparing samples between gels.

A sub-set of clearly visible bands was excised from multiple gels to undergo sequencing. PCR was performed on eluted bands using the same procedure as above. After purification using a QIAquick PCR Purification Kit (Qiagen), a sequencing reaction

of 10 μL was set up with 25-50 ng of DNA, 0.3 μM of the unlabeled 341f primer (Table 2.4), and 4 μL of BigDye™ (Applied Biosystems, Foster City, CA). Product was purified on a G-50 Sephadex (Sigma) column, denatured in formamide for 2 minutes at 95° C, cold-shocked on ice for 30 seconds, and analyzed on an ABI 310 Prism (Applied Biosystems). Phylogenetic analyses were conducted by aligning the 16S rDNA sequences with those of highest BLAST values using the Genetic Computer Group (GCG) package (Madison, WI).

3. *Chitinase Activity*

The activity of chitinase in the bacterial cultures was determined using a slight variant of the Montgomery and Kirchman (1993) protocol. The substrate methylumbelliferyl-diacetyl-chitobioside (MUF-DC; Sigma) was used in culture incubations to determine the relative amount of chitinase activity in each bacterial culture. Fifty μL of MUF-DC was added to 5 mL of sample, for a final MUF-DC concentration of 500 μM . Each sample was incubated on a shaker table at room temperature for 1 to 4 hours. The optimum duration of incubation was determined by analyzing a time course of MUF-DC degradation to the time interval over which MUF-DC degradation was linear. The enzymatic reaction was stopped with 0.5 mL of a fixative (50 mM glycine; 200 mM ammonium hydroxide, pH 10.5). These killed samples were then frozen at -20° C until measured.

The fluorescence of the methylumbelliferone (MUF) released in each sample was measured using a fluorometer (Hoefer; excitation 365 nm \pm 7 nm; emission filter 460 nm \pm 15 nm). The fluorometer was zeroed against 1.9 mL of carbonate buffer (0.20 M) then 100 μL of sample was added and MUF fluorescence was quantified. A standard curve

relating concentration of free MUF (Sigma) to fluorescence was determined prior to each incubation and used to convert fluorescence to free MUF concentration, which was then used to calculate MUF-DC hydrolysis rate.

V. Statistics

Unless denoted otherwise, statistical analyses were performed using the SPSS 11.0 package (SPSS Inc.; Table 2.6). Tests for linearity and zero-slope were performed using regression curve tests of both Linear and Quadratic ANOVA models. If the Quadratic model was significant ($p\text{-value}_{\text{linear}} \leq 0.05$), then the trend was considered to be non-linear. If the Quadratic model was insignificant ($p\text{-value}_{\text{linear}} > 0.05$), the trend was linear. Once the trend was found to be linear, the slope was non-zero if the Linear model was significant ($p\text{-value}_{\text{zero}} \leq 0.05$).

To compare two trends (two slopes), a small-sample t-test for parallelism was performed using the following test statistic:

$$T = \frac{\beta_1 - \beta_2}{S_{\beta_1 - \beta_2}}$$

Where β_1 is the slope for treatment 1, β_2 is the slope for treatment 2, and $S_{\beta_1 - \beta_2}$ is the standard deviation estimate of the slope differences. The standard deviation requires that the estimated variances of each slope be pooled in the following manner:

$$S^2_{\beta_1 - \beta_2} = S^2_{\text{pooled}} \left[\frac{1}{(n_1 - 1)S_1^2} + \frac{1}{(n_2 - 1)S_2^2} \right]$$

and

$$S^2_{pooled} = \frac{(n_1 - 2)E_1^2 + (n_2 - 2)E_2^2}{n_1 + n_2 - 4}$$

Where S^2_{pooled} is the pooled variance, S^2 is the individual variance, and E^2 is the residual mean-square error. The test statistic was used to calculate a p-value based upon the pooled degrees of freedom:

$$df = n_1 + n_2 - 4$$

The two trends were considered to be significantly different when the $p\text{-value}_{\text{slope}}$ was ≤ 0.05 .

The last type of statistical analysis performed was the t-test for comparing means of independent samples. Two means were considered to be significantly different when the $p\text{-value}_t$ was ≤ 0.05 .

Table 2.1: Fungal Stage Treatments. Leaves in each treatment were sterilized and inoculated using the following experimental design.

Experiment	Treatment	Type of Sterilization	Fungal Inoculation
I	Empty	(no leaves)	-
I	Natural	No sterilization	-
II	Control	Autoclave	-
I, II	PS	Autoclave	<i>Phaeosphaeria spartinicola</i>
I	MS	Autoclave	<i>Mycosphaerella</i> sp. 2
I	PS+MS	Autoclave	<i>Phaeosphaeria spartinicola</i> and <i>Mycosphaerella</i> sp. 2

Table 2.2: Bacterial Stage Treatments. Rinse water collected from the Fungal Stage was added to the bacteria cultures using the following experimental design.

Treatment	Type of Rinse Added to Bacterial Cultures	Additional Substrate
(P+D)	Particulate and Dissolved (Not Filtered)	-
(D)	Dissolved (Filtered)	-
(D+C)	Dissolved (Filtered)	Chitin

Table 2.3: Inoculation and sampling timeline. Standing dead *Spartina* leaves were collected from Dean Creek on Sapelo Island, GA in November, 2001 and May, 2002.

Experiment	Leaf Inoculation Date	First Sampling (Day 0)	Midpoint Sampling	Final Sampling
I	November 13, 2001	November 20, 2001 (Day 0)	December 6, 2001 (Day 16)	December 22, 2001 (Day 32)
II	May 27, 2002	June 2, 2002 (Day 0)	June 14, 2002 (Day 12)	June 26, 2002 (Day 24)

Table 2.4: Sequences of primers used in this study. The GC-clamp was added to the 341f primer and used with 534r to amplify ~200 bases from the variable 3 region of eubacterial 16S rRNA genes for DGGE analysis. ITS 1F and ITS4A were used to amplify ~600 bases from the ITS 1, 5.8S, and ITS2 regions of ascomycete 16S rRNA genes for TRFLP analyses.

Primer	Sequence (5'→3')	Manufacturer
341f	CCTACGGGAGGCAGCAG	Operon
534r	FI ^a ATTACCGCGGCTGCTGG	Operon
GC-Clamp	CGCCCGCCGCGCCCGCGCCCGTCCCG CCGCCCGGCC	Operon
ITS 1F	F ^b CTTGGTCATTTAGAGGAAGTAA	Applied Biosystems
ITS4A	CGCCGTTTACTGGGGCAATCCCTG	Applied Biosystems

^aFI, fluorescein label

^bF, FAM label

Table 2.5: DNA used for DGGE Lane Standards. The lane standard consisted of equal molar concentrations of PCR products from the following organisms. Clones were selected to provide bands to cover the entire 45 – 65% denaturing gradient.

Standard	Supplier/Location
<i>Clostridium perfringens</i>	Sigma
<i>Bacillus thuringiensis</i>	Sigma
4 clones*: ML617.5J-23 ML617.5J-22a** ML617.5J-35 ML617.5J-1	Mono Lake, California

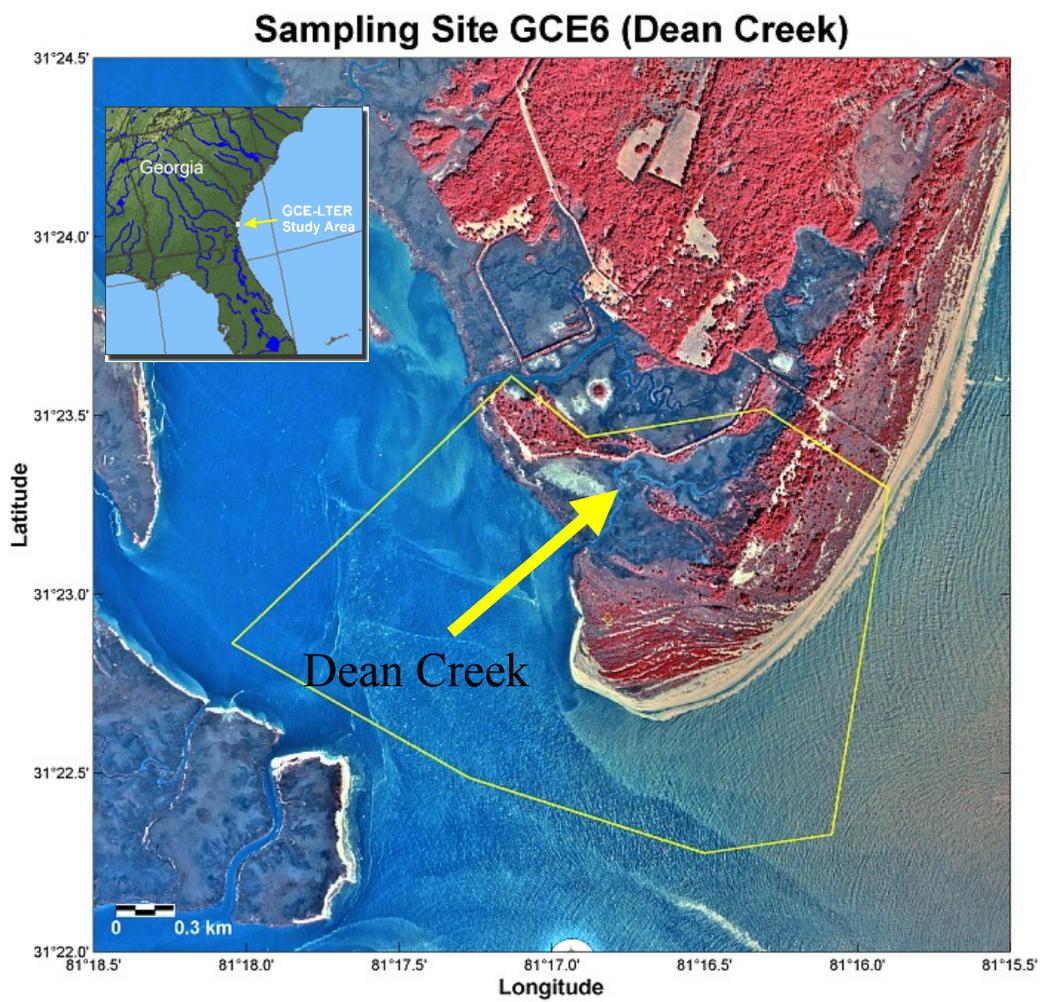
* All clones are referenced in Humayoun et al. 2003.

** Not submitted to GenBank, but it is 99% similar (299/300 bp) to clone ML617.5J-13 (AF507825)

Table 2.6: Summary table of p-value notation used in this thesis. The notation associated with each test is described along with what a significant p-value for each test indicates. All p-values were generated from SPSS software.

P-value notation	Type of test performed	Null Hypothesis	When P-value ≤ 0.05 ...
P-value _{linear}	ANOVA; quadratic function to test for linearity	H ₀ = Linear slope	Trend is non-linear
P-value _{zero}	ANOVA; linear function to test for a significant slope	H ₀ = Zero slope	Trend has a non-zero slope
P-value _{slope}	t-test; trend comparison by evaluating slopes	H ₀ = Equal slopes	The two slopes are significantly different from each other
P-value _t	t-test; comparison of mean values	H ₀ = Equal means	The two treatments have significantly different means

Figure 2.1: Map of Sapelo Island Sampling Site



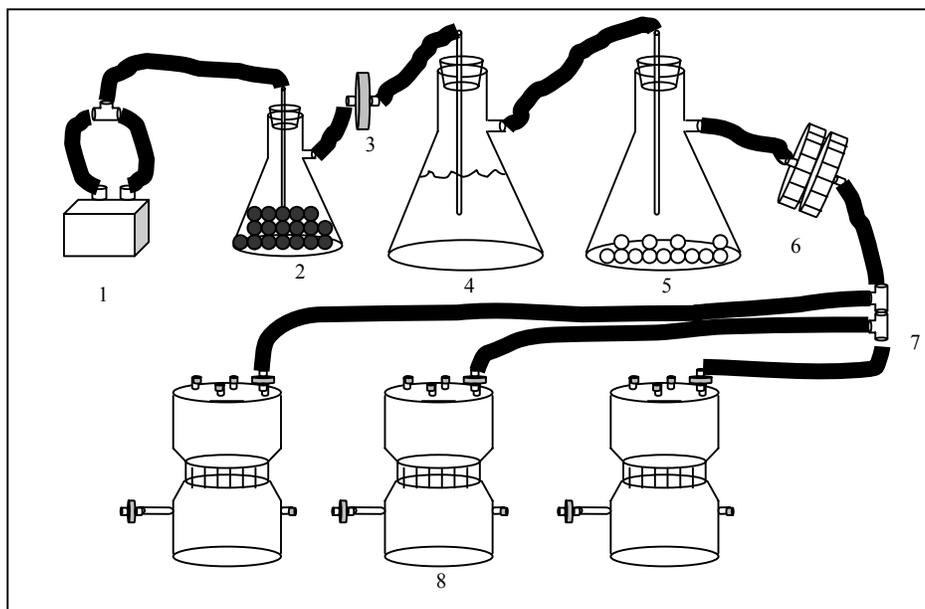


Figure 2.2: Air Flow Diagram. Numbers represent different steps in the air filtration system. (1) An aquarium air pump (Aqua Culture) was used to aerate the microcosms. The outlets of the pump were combined with tubing to maximize the amount of airflow, up to 2800 cm^3 per minute. Impurities were removed from the air by passing it through (2) activated charcoal (Sigma) and (3) an autoclaved in-line filter ($0.2 \mu\text{m}$ PTFE hydrophobic vent filter; Millex, Millipore; airflow rate $\geq 15 \text{ L} \cdot \text{min}^{-1}$ at 0.35 kg/cm^2). The air was humidified by (4) bubbling it through $0.22 \mu\text{m}$ filtered Milli-Q water then passed through (5) a flask containing 225 g of 5 mm glass beads (Fisher) to increase surface area (Experiment I only) to trap droplets. (6) Finally, the air passed through another filter (HA $0.45 \mu\text{m}$ filter; Millipore) held in a 47 mm Swinnex in-line filter holder (Millipore). (7) Then the air was split into three directions using plastic connectors. (8) This air was then distributed to three Fungal Microcosms constructed using Sterifil® 47 mm aseptic systems (Millipore). Grid sizes of the Sterifil® filter holder were variable ($0.25 \text{ mm}^2 - 0.32 \text{ mm}^2$).

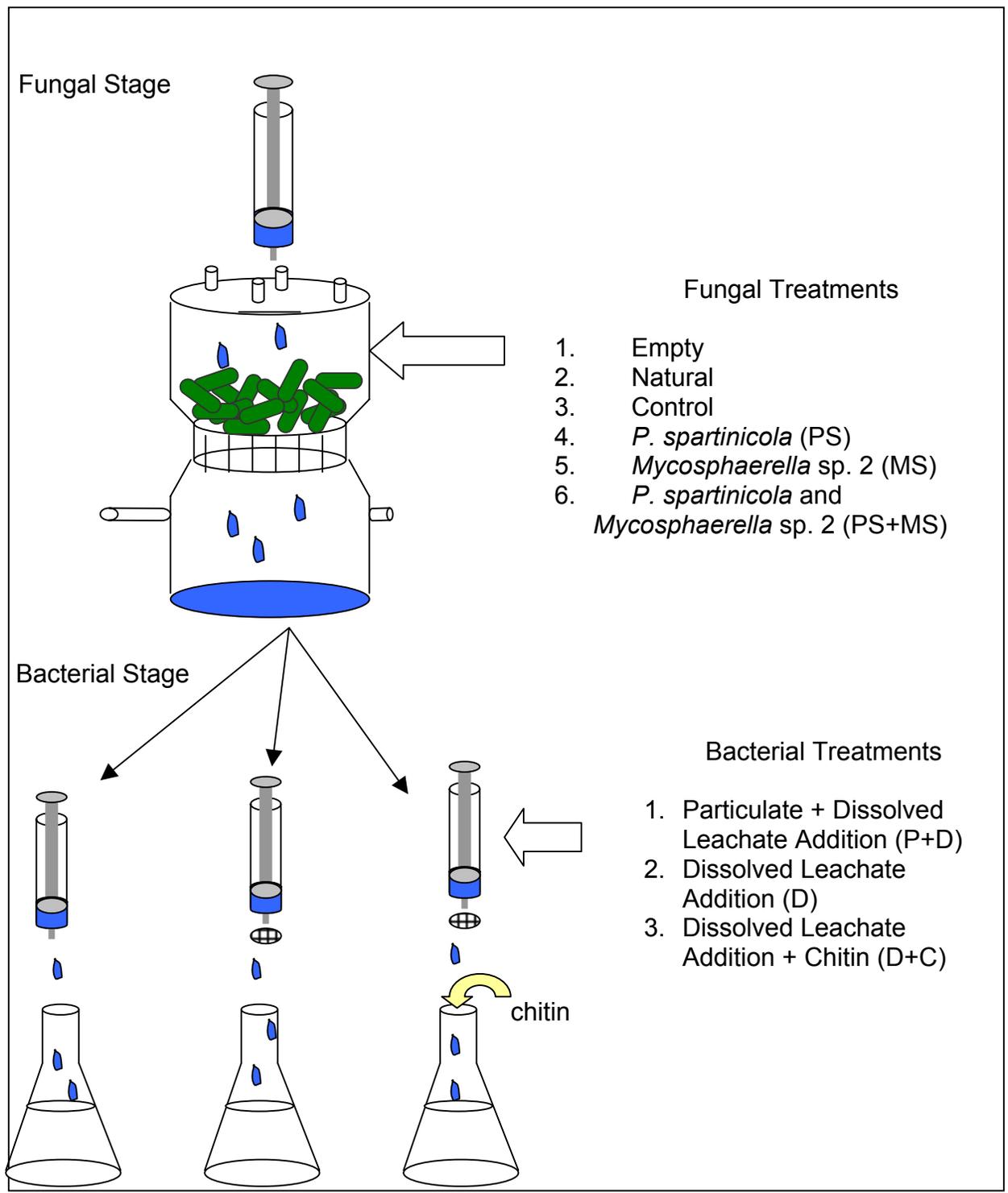


Figure 2.3: Diagram of Fungal and Bacterial Stages. In the fungal stage, *Spartina alterniflora* leaves were inoculated with fungi according to the experimental design. Dean Creek water was added to the Fungal Stage every four days through a top rubber port. The rinse water was collected and distributed to the Bacterial Stage according to the experimental design.

CHAPTER 3

RESULTS

Fungal growth was observed as aerial hyphae in the microcosms within a week of inoculation. In Experiment I, white/tan, robust hyphae were visible first in the *P. spartinicola* (PS) treatment then in the combined fungal treatment (PS+MS). A less robust and white/gray hyphal growth was noted in the *Mycosphaerella* (MS) treatment. Growth of aerial hyphae was less notable in the Natural treatment. Due to the presence of hyphae, data from the Control treatment (sterile leaf control) from Experiment I are omitted. In general, all hyphae remained visible throughout the experiment but hyphal mass appeared to decline over time.

In Experiment II, white/tan, robust hyphae were again visible in the *P. spartinicola* (PS) treatment within four days of inoculation. The hyphae remained throughout the experiment, but hyphal mass again appeared to decline over time. Black ascospores were visible on these leaves when the microcosms were taken apart 29 days after inoculation. No visible aerial hyphae were detected in the Control treatment from Experiment II.

I. Leaves Inoculated with Fungi

A. Ergosterol Content

In Experiment I, ergosterol content of most Leaf treatments (all treatments excluding Empty) ranged from 422 to 923 $\mu\text{g}\cdot\text{g}$ of ash free dry weight (AFDW)⁻¹ (Table

3.1). The only exception was in the PS treatment where ergosterol increased from 472 ± 71 to $2464 \mu\text{g}\cdot\text{g AFDW}^{-1}$ between days 0 – 23. All treatments showed increasing ergosterol content with time, but these changes were not significant (Table 3.1). The PS treatment was the only one to have a non-linear rate of ergosterol change ($p\text{-value}_{\text{linear}} < 0.01$). It should be noted that several samples from Experiment I lost volume during shipping.

During Experiment II, ergosterol content ranged from 233 to $1057 \mu\text{g}\cdot\text{g}^{-1}$ AFDW and varied significantly between treatments ($p\text{-value}_t < 0.01$; Table 3.1). In the Control treatment, ergosterol content remained low throughout the experiment ($322 \pm 98 \mu\text{g}\cdot\text{g AFDW}^{-1}$) and did not increase over time ($p\text{-value}_{\text{zero}} > 0.05$). However, ergosterol increased significantly in the PS treatment ($26 \mu\text{g}\cdot\text{g}^{-1}$ AFDW $\cdot\text{day}^{-1}$; $p\text{-value}_{\text{zero}} < 0.001$). The rate of increase in this treatment was significantly greater than in the Control treatment and in Experiment I ($p\text{-value}_{\text{slope}} < 0.001$; Table 3.1).

B. Fungal Community

TRFLP profiles for each microcosm confirmed that the inoculated fungi were indeed present in the leaves throughout the experiments. In the Natural treatment, overall species composition did not change during the experiment. Ascomycetes present on Natural leaves included *P. spartinicola*, *Mycosphaerella* sp. 2, *P. halima*, and '4clt', an ascomycete described in Buchan et al. (2002)

The Control treatment in Experiment I was contaminated by *P. halima*. However, in the Control for Experiment II, fungi were never observed. PCR was unsuccessful in the second Control.

C. Organic Density, Percent Ash

Organic density (OD) of the leaves in all treatments did not vary significantly during the experiments ($p\text{-value}_{\text{zero}} > 0.05$), nor did average OD differ between treatments ($p\text{-value}_t > 0.05$; Tables 3.1 and 3.2). The initial densities, 10.1-11.3 $\text{mg}\cdot\text{cm}^{-2}$, decreased to 8.3-8.6 $\text{mg}\cdot\text{cm}^{-2}$ in Experiment I and to 7.1-7.4 $\text{mg}\cdot\text{cm}^{-2}$ in Experiment II.

Ash content of the leaves did not change during Experiment I; however, ash content increased during Experiment II ($p\text{-value}_{\text{zero}} < 0.01$; Table 3.1). The ash content averaged 32.7 ± 7.9 % in Experiment I while increasing from 18 % to ~40 % in Experiment II. No significant difference was found between treatments within each experiment.

D. Leaf Carbon and Nitrogen

In Experiment I, leaf carbon content decreased significantly over time in the Natural, PS, and PS+MS treatments ($p\text{-value}_{\text{zero}} < 0.05$; Table 3.1). Leaf carbon was initially 42.1 to 43.7 % of leaf dry weight (DW) before decreasing to 34.2 to 38.4 %. Rates of carbon loss were statistically similar in the Natural, PS, and PS+MS treatments (-0.2 % \cdot day⁻¹; $p\text{-values}_{\text{slope}} < 0.05$).

In Experiment II, leaf carbon content was initially 43.3 %. In the PS treatment, carbon content declined at a rate of -0.2 % \cdot day⁻¹ ($p\text{-value}_{\text{zero}} < 0.001$). However, in the Control treatment, leaf carbon content did not change over time. Average carbon content was significantly higher in the Control than in the PS treatment ($p\text{-value}_t < 0.05$).

In Experiment I, leaf nitrogen content did not vary significantly, ranging between 0.5 and 0.6 % of leaf DW in all treatments (Table 3.1). In Experiment II, leaf nitrogen fluctuated but exhibited a net decrease by day 29. Even so, nitrogen values only

ranged from 0.6 to 0.7 %. Average nitrogen did not vary significantly between Fungal treatments (PS, MS, PS+MS) of either experiment, but the nitrogen content was significantly higher in the Natural treatment than in the PS+MS treatment ($p\text{-value}_t < 0.05$; Table 3.2).

Carbon to nitrogen ratios (C:N; w/w) of the leaves typically declined by the endpoint of each experiment; however, the data were variable (Table 3.1). In Experiment I, C:N (w/w) values ranged between 65 and 83 and generally did not change over time. Only the rate of C:N decrease in the Natural treatment was significant ($p\text{-value}_{\text{zero}} < 0.001$; Table 3.1). In Experiment II, C:N (w/w) ratios ranged between 57 and 73 but did not change significantly. In addition, C:N (w/w) was not different between treatments ($p\text{-value}_t > 0.05$).

II. Particulate Material Released from Decaying Leaves

A. Particle Quantity

The small amount of particles collected from the Empty treatment did not change throughout the experiment ($p\text{-value}_{\text{zero}} > 0.05$); therefore, data from the Empty treatment served as a blank and were subtracted from the remaining treatments. In Experiment I, the amount of particles rinsed from the leaves ranged between 0.6 to 33.7 mg and generally increased over time (Figure 3.1.I). The Natural treatment released particles at a significantly higher rate than Fungal treatments ($p\text{-value}_{\text{slope}} < 0.001$), which all had similar patterns of particle release ($0.7 \text{ mg}\cdot\text{day}^{-1}$ versus $0.2 \text{ mg}\cdot\text{day}^{-1}$ respectively).

Since a blank (Empty treatment) was not run in Experiment II, we used the average value from Experiment I Empty treatments as an estimate of background particles. In Experiment II, more particles were released from the uninoculated Control

than from the PS treatment ($p\text{-value}_t < 0.001$; Figure 3.1.II). In addition, particle release increased the Control at a rate of $0.7 \text{ mg}\cdot\text{day}^{-1}$ ($p\text{-value}_t < 0.05$), while decreasing in the PS treatment. The small amount of particles released from PS decreased significantly over time ($-0.1 \text{ mg}\cdot\text{day}^{-1}$; $p\text{-value}_{\text{zero}} < 0.05$).

By summing the weights of the particles recovered, an estimate of total amount of particulate material lost from each treatment was calculated over the course of the experiment (Table 3.3). Particle loss was greatest for the Natural treatment ($146.3 \pm 28.0 \text{ mg}$) while Fungal treatments lost an average of $60.1 \pm 27.4 \text{ mg}$. In Experiment II, the Control lost $105.6 \pm 12.5 \text{ mg}$ of particles while the PS treatment lost $15.6 \pm 5.1 \text{ mg}$.

B. Particle Carbon and Nitrogen

Average amounts of particulate carbon lost were highly variable for most treatments in Experiment I (Table 3.4). Carbon loss averaged 1.4 to 8.0 mg in all treatments. In Experiment II, average particulate carbon loss ranged from 0.3 to 6.6 mg and was higher in the Control than in the PS treatment. In addition, the amount of particulate carbon lost from the Control increased over time ($0.7 \text{ mg}\cdot\text{day}^{-1}$; $p\text{-value}_{\text{zero}} < 0.05$) but did not change for the PS treatment.

The concentration of carbon within the particles was also highly variable for most treatments in Experiment I (Figure 3.2.I-A). Carbon concentrations ranged from 11.7 to 85.8 % of particle DW in all treatments. Particle carbon concentrations only increased significantly in the PS+MS treatment ($1.3 \% \cdot \text{day}^{-1}$; $p\text{-value}_{\text{zero}} < 0.05$). In Experiment II, carbon concentrations ranged from 4.6 to 69.6 % of particle DW and were significantly higher in the Control than in PS ($p\text{-value}_t < 0.01$; Figure 3.2.II-A). Particulate carbon concentrations did not significantly increase or decrease during Experiment II.

Average particulate nitrogen losses were variable between sampling points for most treatments in Experiment I (Table 3.4). At each rinse, the amount of nitrogen lost ranged from 0.2 to 1.0 mg in all treatments. In Experiment II, average particulate nitrogen loss ranged from 0.0 to 0.6 mg and was higher in the uninoculated Control treatment than in the PS treatment ($p\text{-value}_t < 0.01$). As with carbon, the average amount of particulate nitrogen lost from the Control increased over time, but this increase was not linear ($p\text{-value}_{\text{linear}} < 0.05$).

The concentration of nitrogen within the particles was also highly variable in Experiment I (Figure 3.2.I-B). Nitrogen concentrations ranged from 1.4 to 15.5 % of particle DW in all Leaf treatments while averaging 1.2 ± 0.4 % in the Empty treatment. Particle nitrogen content only increased significantly in the PS+MS treatment ($0.2 \text{ \%} \cdot \text{day}^{-1}$; $p\text{-value}_{\text{zero}} < 0.05$). In Experiment II, nitrogen content ranged from 0.7 to 13.4 % of particle DW and were significantly higher in the Control than in PS ($p\text{-value}_t < 0.01$; Figure 3.2.II-B). Particulate nitrogen content significantly increased during Experiment II.

Carbon to nitrogen ratios (w/w) for particles ranged from 4 to 15 in Experiment I and 5 to 17 in Experiment II (Figure 3.2.C; Table 3.4). Throughout Experiment I, particle C:N (w/w) did not change in the Natural treatment (8 ± 1), while C:N significantly decreased in all Fungal treatments (PS, MS, PS+MS). Rates of C:N decrease did not differ between treatments of either Experiment. C:N ratios were initially highest in the Fungal treatments for Experiment I. In Experiment II, particles from the PS treatment generally had lower C:N than the uninoculated Control ($p\text{-value} < 0.05$).

C:N (w/w) values of particles were averaged over 32 days in Experiment I and over 24 days in Experiment II (Table 3.4). Fungal treatments (PS, MS, PS+MS) showed comparable, statistically significant higher values (8 ± 3 , 8 ± 3 , 9 ± 3 respectively) than the Natural treatment (8 ± 1). In Experiment II, particles from the PS treatment were significantly more enriched in nitrogen than those from the Control treatment (10 ± 3 ; 13 ± 4 respectively; $p\text{-value}_t < 0.05$).

III. Leachate from Decaying Leaves

A. Dissolved Organic Carbon (DOC)

DOC in the leachate was elevated in the presence of leaves and fungi (Figure 3.3.A). Of the Leaf treatments, Natural leachate contained the lowest and most consistent DOC concentrations in Experiment I, but DOC concentrations decreased throughout the experiment (average 2.5 ± 0.5 mM; $p\text{-value}_{\text{zero}} < 0.001$; Figure 3.3.I-A). Fungal treatment leachate (PS, MS, PS+MS) contained the highest DOC concentrations on average, and these concentrations decreased non-linearly throughout Experiment I ($p\text{-value}_{\text{linear}} < 0.01$). DOC concentrations in Fungal treatment leachate was initially higher in Experiment I (~30 – 45 mM) than in Experiment II (~26 mM; Figure 3.3.II-A); but DOC concentrations were similar for both Experiments by Day 24 (~5 mM). In all Fungal treatments, the majority of the DOC was lost during the first 0-8 days. When comparing the treatments of Experiment II, average DOC concentrations in leachate from Control treatments were higher than in the PS treatment ($p\text{-value}_t < 0.001$; Figure 3.3.II-A), but rates of DOC loss were higher in PS than the Control ($-464.2 \mu\text{M}\cdot\text{day}^{-1}$; $p\text{-value}_{\text{slope}} < 0.001$).

B. Total Dissolved Nitrogen (TN)

Total dissolved nitrogen in the leachate was elevated significantly in treatments containing leaves or fungi (N, PS, MS, PS+MS, C), similar to DOC trends (Figure 3.3.B). The Natural leachate had the lowest and most consistent TN concentrations in Experiment I ($\bar{x} = 83.0 \pm 20.0 \mu\text{M}$; Figure 3.3.I-B). TN concentrations in leachate from Fungal treatments of both Experiments were high initially (970.4 - 1134.2 μM) then decreased non-linearly to 62 - 234 μM with time ($p\text{-value}_{\text{linear}} < 0.05$). In Experiment II, TN concentration was initially highest in the *P. spartanicola* treatment; however, TN concentration in uninoculated Control leachates was higher than in PS leachate after 8 days (Figure 3.3.II-B). Rates of TN decrease were linear in the Control treatment ($p\text{-value}_{\text{linear}} > 0.05$; $-11.31 \mu\text{M}\cdot\text{day}^{-1}$) while non-linear in PS ($p\text{-value}_{\text{linear}} < 0.05$).

C. Total Dissolved Phosphorus (TP)

Leached total phosphorus was also elevated in treatments with leaves and fungi (Figure 3.3.C). The Natural and Fungal treatments leached similar quantities with similar time courses of TP concentration in rinse water. Leached TP was initially high (63.3-197.6 μM) then decreased to 18.6-76.0 μM after 24 days. Leached TP concentration decreased rapidly between days 0-12 then stabilized. This trend is more pronounced in Experiment II than in Experiment I. When comparing the two treatments of Experiment II, leached TP concentration was initially similar for both (~ 146 to $170 \mu\text{M}$; Figure 3.3.II-C). However, by day 8 the PS treatment leached significantly less TP than the Control. This difference was maintained over the remaining 16 days of the experiment. The decline in rates of TP loss from the Control treatment was linear ($-7.4 \mu\text{M}\cdot\text{day}^{-1}$; $p\text{-value}_{\text{linear}} > 0.05$).

D. Stoichiometry of Leached Carbon, Nitrogen, and Phosphorus

Concentrations of carbon, nitrogen, and phosphorus in leachate were used to calculate Carbon:Nitrogen (C:N) and Nitrogen:Phosphorus (N:P) ratios (mole/mole). Average molar C:N in Experiment I ranged from 30 to 45 and was variable between sampling days (Figure 3.4.I-A; Table 3.5). There was no significant difference of C:N ratios between treatments, but several samples in the PS+MS treatment produced higher C:N ratios than other treatments. On average, C:N ratios were lowest for the MS treatment (30 ± 8) and highest in the PS+MS treatment (45 ± 18 ; Table 3.5).

In Experiment II, the average C:N ranged from 25 ± 7 in PS to 41 ± 5 in the Control (Figure 3.4.II-A; Table 3.5). A major change in the C:N ratio of leachate for both treatments was detected on day 4, but apart from that sample, C:N of the leachate was relatively constant over time ($p\text{-value}_{\text{zero}} > 0.05$). On average, C:N was significantly higher in the Control than the PS treatment (Table 3.5; $p\text{-value}_t < 0.001$).

N:P (mole/mole) of leachate was not as variable as C:N; in addition, N:P showed more general trends with time (Figure 3.4.I-B, Table 3.5). In Experiment I, average N:P ranged from 2 to 7. Among the Fungal treatments, N:P decreased over time; in contrast, it increased over time in the Natural treatment (all $p\text{-values}_{\text{zero}} < 0.05$). On average, the lowest leachate N:P occurred in the Natural treatment (2 ± 1 ; Table 3.5). Of the Fungal treatments, PS produced leachate with a lower N:P than either the MS or PS+MS treatment (respectively: 5 ± 3 ; 6 ± 1 ; 7 ± 3).

In Experiment II, average N:P ranged from 3 to 13 (Figure 3.4.II-B, Table 3.5). Values were highly variable in the Control (13 ± 10), increasing rapidly between day 4-8

before slowly decreasing. In contrast, N:P in the PS treatment was fairly constant and considerably lower (3 ± 2 ; $p\text{-value}_t < 0.01$; Table 3.5).

E. Total Dissolved Carbohydrates

Total carbohydrates made up $< 1\%$ of the material dissolved in leachate. Most of the glycosyl residues in the carbohydrate fraction were hexitols (≥ 40 mole %; Figure 3.5). The remaining pool of residues came from neutral sugars that included arabinose, galactose, glucose, mannose, and xylose. Arabinose was only detected in the Control treatment, but it was absent by day 24. Galactose, while present in each PS sample, disappeared from the Control by day 24 as well. Glucose and mannose were present in the Control and increased in relative concentration with time (10.4 to 14.8 mole %, 5.5 to 13.4 mole % respectively). The fraction of xylose in the Control varied considerably over time. In the PS treatment, glucose, mannose, and xylose all increased in concentrations relative to other sugars with time (9.4 to 17.6 mole %; 3.1 to 10.0 mole %; 2.0 to 7.7 mole % respectively). Galactose was also present in PS, but its relative concentration varied considerably.

The total glycosyl residue composition (glycosyl residues from hexitols and neutral sugars combined) from Experiment II is shown in Figure 3.6. These residues include arabinose, galactose, glucose, mannose, rhamnose, and xylose. Rhamnose, not detected as a neutral sugar, was present in low mole percent in all Control samples, but it was only present in PS on day 24. Throughout Experiment II, arabinose and mannose both decreased in the Control while galactose, rhamnose, and xylose increased. The relative concentration of glucose in the Control was constant.

Arabinose decreased over time in the PS treatment, while galactose increased. No trend was noted in the relative proportions of glucose, mannose, and xylose in PS.

IV. Bacterial Cultures Receiving Leachate Additions

A. Bacterial Counts

The number of bacteria in both Experiments was high ($\sim 10^7$ bacteria·mL⁻¹) and did not vary significantly between treatments (Figure 3.7). Bacteria responded positively to initial leachate additions. In Experiment I, rapid growth occurred between days 0-4 (Figure 3.7.I). In particular, Particulate + Dissolved (P+D) additions produced higher bacterial numbers. Between days 4-6, however, the bacterial populations declined to abundances comparable to initial bacterial counts. After day 6, numbers slowly increased through the remainder of the incubation.

In Experiment II, bacteria also responded positively to initial leachate, but rapid growth only occurred between days 0-2 with smaller increases than seen in Experiment I (Figure 3.7.II). Bacteria abundance then decreased until day 6 when numbers began to slowly increase. Overall, bacterial numbers in Experiment II did not change significantly over time.

B. Bacterial Community

Bacterial community composition based upon DGGE banding pattern alone did not indicate a treatment effect. Figures 3.8 and 3.9 display dendrograms of Midpoint and Endpoint bacterial populations of both Experiments based upon Molecular Analyst® software using UPGMA clustering algorithms and the Jaccard band similarity coefficient. All of the similarities between populations were below 80% at each time point except for the Midpoint similarity between the D and D+C Natural treatments.

Because of the low similarity between whole-community banding patterns, we also looked at the distribution of individual operational taxonomic units (OTUs) between treatments. Each band position was treated as an individual OTU as described by Ferrari and Hollibaugh (1999). By aligning all gels, OTUs were designated numbers based upon their position. Tables 3.6 and 7 show the presence and absence of individual OTUs in DGGEs from each treatment. By this method, some OTUs appear more frequently than others. For example, OTUs corresponding to positions 275 and 289 appear in all samples in both experiments. However, OTUs such as 282 only occur in Experiment II, and OTU 223 occurs exclusively in Experiment I. Certain OTUs are absent from particular treatments as well. For example, OTUs 74-166 are never identified in the MS treatment. Similarly, OTUs 136-191 were not found in the PS treatment from Experiment II.

Table 3.8 identifies dominant OTUs that were found in > 3 bacteria treatments; the data are collapsed so that the presence/absence of OTUs is shown for in each treatment. Among the Fungal treatments in Experiment I, OTUs 208 and 236 are present in PS and MS but not in PS+MS; OTUs 216 and 269 are present in MS and PS+MS but not PS; while OTUs 100 and 166 are present in PS and PS+MS but not MS. Also, OTUs 216 and 236 are absent from Particulate + Dissolved treatments; and OTUs 166, 178, 260, and 269 are absent from the Dissolved + Chitin treatments.

Most bands cut from DGGE gels were sequenced successfully to ~100 base pairs (Table 3.9), allowing us to identify OTUs and to insure that a single OTU located on different gels corresponds to the same sequence. Whenever possible, several bands from the same OTU position were sequenced and compared. With one

exception, all bands from the same position had exactly the same sequence. This exception was found in OTU 226 where 4 out of 5 of the bands sequenced matched, but the 5th band (D6) did not.

Using this sequence data, bacteria were classified minimally to the phylum level by > 90% sequence identity and < 10^{-12} error (Table 3.9). Bacteria from α -Proteobacteria; *Cytophaga*, *Flavobacterium*, *Bacteroides* (CFB); γ -Proteobacteria; and High G+C Gram positive groups were found in these experiments. The most ubiquitous OTUs, 275 and 289, represent α -Proteobacteria. Other common OTUs such as 226, 200, and 166 correspond to CFBs. High G+C content Gram-positive bacteria (OTU 269) were only found in MS, PS+MS, and the Control treatments. Similarly, γ -Proteobacteria (OTUs 178, 191) were not detected in the Natural and PS treatments.

OTU richness as measured by Margalef's diversity index (D_{MG}) using data on OTU distributions is shown in Figure 3.10. The greatest diversities were found in Experiment II with D_{MG} values reaching 0.33. Leachate additions (D) to bacterial cultures produced greater OTU richness compared with P+D and D+C additions. In Experiment I, PS+MS produced the greatest D_{MG} of the leaf treatments while the D_{MG} from MS treatments was much lower on average (Figure 3.11). In Experiment II, average OTU richness was the same in the PS and Control treatments.

C. Chitinase Activity

Chitinase activity varied between treatments and time points (Figure 3.12). In Experiment I, more chitinase activity was detected in Leaf treatments (Natural, PS, MS, PS+MS) than in the Empty treatment. Chitinase activity in Empty treatments was originally low (~ 7 to $12 \text{ nM}\cdot\text{hour}^{-1}$) and decreased significantly over time with little

difference in average activity between the P+D, D, and D+C additions (Table 3.10). In the Natural treatment, D+C additions initially increased chitinase activity; however, there was no difference in average chitinase activity between the Natural P+D and D+C addition treatments.

Different trends of activity were found in the PS, MS, and PS+MS treatments. The greatest chitinase activity in Experiment I was measured in treatments when both fungi were present alone (PS+MS; Figure 3.12.I). In PS+MS D and D+C treatments, chitinase activity was initially low ($\sim 12 \text{ nM}\cdot\text{hour}^{-1}$), peaked at the 16-day sampling point, then decreased by day 32. The largest activity and variability were measured in the PS+MS P+D and D+C addition treatments. In MS, there was no significant difference in average activity between P+D, D, and D+C additions. In the PS treatments, midpoint chitinase activity was double that of initial activity; on day 32, activity dropped once again. Within all Fungal treatments (PS, MS, PS+MS), average chitinase activity did not differ with the various substrate additions (P+D, D, D+C; Table 3.10).

In Experiment II, chitinase activity was similar to that in Experiment I (Figure 3.12.II). For the Control treatments, activity was initially low ($\sim 7 - 8 \text{ nM}\cdot\text{hour}^{-1}$) and increased by more than 6-fold on day 12. Activity levels either decreased or did not change on day 24; however, different additions (P+D, D, D+C) did not significantly alter average chitinase activity (Table 3.10). Similar to the Control treatments, chitinase activity in the PS treatments was initially low ($\sim 4 \text{ nM}\cdot\text{hour}^{-1}$) and increased by more than 4-fold on day 12. On day 24, chitinase activity was slightly lower in the D+C treatment; in contrast, activity significantly increased with P+D and D addition ($p\text{-value}_{\text{zero}} < 0.05$).

Average chitinase activity in Experiment II was higher in the Control than in the PS treatments.

Table 3.1: Properties of the cut, standing dead *Spartina alterniflora* leaves used in the experiments. Ex = experiment number; Treat = Treatment where N is Natural, PS is *P. spartinicola*, MS is *Mycosphaerella* sp. 2, PS+MS is *P. spartinicola* and *Mycosphaerella* sp. 2, and C is Control; Day = number of days following fungal inoculation; Ergosterol was measured in $\mu\text{g}\cdot\text{g}^{-1}$ AFDW; Organic Density was measured in $\text{mg}\cdot\text{cm}^{-2}$; % Ash = weight percent of ash in dry leaves; Carbon and Nitrogen were measured in % of leaf DW^{-1} ; C:N was calculated by (w/w) carbon:nitrogen.

Ex	Treat	Day	Ergosterol ($\mu\text{g}\cdot\text{g}^{-1}$ AFDW)	Organic Density ($\text{mg}\cdot\text{cm}^{-2}$)	% Ash	Carbon (% of DW)	Nitrogen (% of DW)	C:N (w/w)	
I	N					*↓		*↓	
		0	625 ± 9 (2) ^a	11.3 ± 2.1 (15)	22.5 ± 5.1 (15)	42.1 ± 0.3 (2)	0.6 ± 0.0 (2)	75 ± 0 (2)	
		23	885 (1)	8.0 ± 1.6 (15)	33.9 ± 4.1 (15)	40.1 ± 0.6 (2)	0.6 ± 0.0 (2)	71 ± 2 (2)	
		39	736 ± 265 (2)	8.4 ± 1.3 (30)	35.4 ± 4.5 (30)	37.8 ± 0.5 (4)	0.6 ± 0.0 (4)	66 ± 1 (4)	
		PS		**			*↓		
			0	472 ± 71 (2)	10.1 ± 1.6 (15)	21.9 ± 5.7 (15)	43.7 ± 0.3 (2)	0.6 ± 0.0 (2)	77 ± 2 (2)
			23	2464 (1)	8.8 ± 1.1 (15)	38.4 ± 3.7 (15)	37.7 ± 1.7 (2)	0.6 ± 0.0 (2)	68 ± 1 (2)
		39	632 ± 214 (2)	8.6 ± 1.2 (30)	37.1 ± 4.7 (30)	38.4 ± 0.8 (2)	0.5 ± 0.1 (2)	73 ± 10 (2)	
		MS	0	472 ± 71 (2)	10.1 ± 1.6 (15)	21.9 ± 5.7 (15)	43.7 ± 0.3 (2)	0.6 ± 0.0 (2)	77 ± 2 (2)
	23		474 (1)	8.1 ± 1.0 (15)	36.9 ± 3.2 (15)	38.9 ± 0.4 (2)	0.6 ± 0.1 (2)	77 ± 3 (2)	
	39		762 ± 74 (2)	8.4 ± 1.3 (30)	35.9 ± 4.1 (30)	34.2 ± 5.9 (2)	0.5 ± 0.0 (2)	74 ± 6 (2)	
	PS+MS					*↓			
		0	472 ± 71 (2)	10.1 ± 1.6 (15)	21.9 ± 5.7 (15)	43.7 ± 0.3 (2)	0.6 ± 0.0 (2)	77 ± 2 (2)	
		23	573 (1)	8.7 ± 1.4 (15)	33.8 ± 4.1 (15)	39.2 ± 0.0 (2)	0.5 ± 0.0 (2)	80 ± 3 (2)	
	39	612 ± 115 (2)	8.3 ± 1.3 (30)	35.2 ± 5.5 (30)	38.4 ± 0.5 (2)	0.5 ± 0.0 (2)	78 ± 7 (2)		
II	PS		*↑		*↑	*↓			
		0	268 ± 24 (3)	10.2 ± 1.5 (15)	17.5 ± 5.8 (15)	43.3 ± 0.0 (2)	0.7 ± 0.1 (2)	63 ± 5 (3)	
		17	726 ± 138 (3)	8.9 ± 1.6 (44)	29.8 ± 9.1 (44)	39.6 ± 0.2 (3)	0.6 ± 0.0 (3)	66 ± 1 (3)	
	29	1024 ± 32 (3)	7.1 ± 1.1 (45)	41.8 ± 2.3 (45)	38.5 ± 1.0 (3)	0.6 ± 0.0 (3)	60 ± 3 (3)		
	C				*↑				
		0	268 ± 24 (3)	10.2 ± 1.5 (15)	17.5 ± 5.8 (15)	43.3 ± 0.0 (2)	0.6 ± 0.1 (2)	63 ± 5 (3)	
17		315 ± 102 (3)	8.4 ± 1.5 (45)	30.1 ± 7.5 (45)	42.3 ± 0.9 (3)	0.6 ± 0.0 (3)	69 ± 2 (3)		
29	377 ± 114 (3)	7.4 ± 1.2 (44)	39.2 ± 3.4 (44)	43.9 ± 4.7 (3)	0.6 ± 0.1 (3)	68 ± 5 (3)			

^a average ± standard deviation (n)

*↑ $P\text{-value}_{\text{zero}} \leq 0.05$ and a significant increase

*↓ $P\text{-value}_{\text{zero}} \leq 0.05$ and a significant decrease

** $P\text{-value}_{\text{linear}} \leq 0.05$ and a non-linear rate

Table 3.2: Average fungal growth and average properties of leaves. Ex = experiment number; Treat. = Treatment where N is Natural, PS is *P. spartinicola*, MS is *Mycosphaerella* sp. 2, PS+MS is *P. spartinicola* and *Mycosphaerella* sp. 2, and C is Control. Ergosterol was measured in $\mu\text{g}\cdot\text{g}^{-1}$ AFDW; Organic Density was measured in $\text{mg}\cdot\text{cm}^{-2}$; % Ash = weight percent of leaf ash; Carbon and Nitrogen were measured in % of leaf DW; C:N was calculated by (w/w) carbon:nitrogen. Averages were calculated over 39 days in Experiment I and 29 days in Experiment II.

Ex	Treat.	Ergosterol ($\mu\text{g}\cdot\text{g}^{-1}$ AFDW)	OD ($\text{mg}\cdot\text{cm}^{-2}$)	% Ash (%)	Carbon (%)	Nitrogen (%)	C:N (w/w)
I							
	N	721 \pm 170 (5) ^b	9.0 \pm 2.1 (60) ^b	32.5 \pm 7.5 (60) ^a	39.4 \pm 2.0 (8) ^a	0.6 \pm 0.0 (8) ^b	69 \pm 4 (8) ^b
	PS	935 \pm 866 (5) ^{ab}	9.0 \pm 1.4 (60) ^b	33.6 \pm 8.3 (60) ^a	39.9 \pm 3.0 (6) ^{ab}	0.6 \pm 0.0 (6) ^{ab}	72 \pm 6 (6) ^{bcd}
	MS	588 \pm 166 (5) ^b	8.7 \pm 1.6 (60) ^{ab}	33.3 \pm 8.0 (60) ^a	38.9 \pm 5.0 (6) ^{ab}	0.6 \pm 0.1 (6) ^{ab}	76 \pm 4 (6) ^{cd}
	PS+MS	548 \pm 98 (5) ^b	8.9 \pm 1.6 (60) ^{ab}	31.5 \pm 7.7 (60) ^a	40.4 \pm 2.5 (6) ^{ab}	0.5 \pm 0.0 (6) ^a	78 \pm 4 (6) ^{cd}
II							
	PS	673 \pm 337 (9) ^b	8.3 \pm 1.8 (104) ^a	33.2 \pm 10.7 (104) ^a	40.1 \pm 2.1 (8) ^a	0.6 \pm 0.0 (8) ^c	63 \pm 4 (8) ^a
	C	332 \pm 98 (9) ^a	8.3 \pm 1.6 (103) ^a	32.0 \pm 9.4 (103) ^a	43.2 \pm 2.7 (8) ^b	0.6 \pm 0.1 (8) ^c	67 \pm 4 (8) ^{abc}

* average \pm standard deviation (n)

^{a,b,c,d} treatments with statistically similar means ($p\text{-value}_t < 0.05$)

Table 3.3: Summed weight of particles collected on GF/F filters by conclusion of experiment. Weights were corrected for blank particle weight (using Empty treatments), and summed over 32 days in Experiment I and 24 days in Experiment II. PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2. A blank (Empty treatment) was not run in Experiment II, so data from Experiment I Empty treatments were used as the blank for particle weights.

Experiment	Treatment	Cumulative Particle Weight (mg)*
I	Natural	146.3 ± 28.0
	PS	59.2 ± 10.8
	MS	55.4 ± 31.0
	PS+MS	72.1 ± 47.3
II	PS	15.6 ± 5.1
	Control	105.6 ± 12.5

* Average ± SD of 2 replicate treatment summations

Table 3.4: Average carbon, nitrogen, and C:N in particles washed from microcosms at each rinse. Data were corrected for background carbon and nitrogen (using Empty treatment as negative control). C:N values were calculated as carbon:nitrogen (w/w). PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2. Data from Experiment I were averaged over 32 days, while data from Experiment II were averaged over 24 days.

Experiment	Treatment	Carbon (mg)*	Nitrogen (mg)	C:N (w/w)
I	Natural	8.0 ± 5.0 ^d	1.0 ± 1.0 ^d	8 ± 1 ^a
	PS	3.4 ± 3.2 ^c	0.5 ± 0.5 ^c	8 ± 3 ^{ab}
	MS	1.4 ± 1.2 ^b	0.2 ± 0.2 ^b	8 ± 3 ^{ab}
	PS+MS	4.3 ± 4.0 ^{cd}	0.5 ± 0.5 ^{cd}	9 ± 3 ^{ab}
II**	PS	0.3 ± 0.3 ^a	0.0 ± 0.0 ^a	10 ± 3 ^b
	Control	6.6 ± 4.8 ^d	0.6 ± 0.5 ^{cd}	13 ± 4 ^c

* average ± standard deviation of two replicate sets of GF/F filters

** no negative control was run during Experiment II, so data from Experiment I Empty treatments were used to subtract out background carbon and nitrogen

a, b, c, d means that were not significantly different ($p\text{-value}_t < 0.05$)

Table 3.5: Average C:N and N:P of dissolved material in *S. alterniflora* leachates. Data were calculated as mole/mole ratios. PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2.

Exp	Treatment	C:N* (mole/mole)	N:P* (mole/mole)
I	Natural	31 ± 9 ^b	2 ± 1 ^a
	PS	35 ± 9 ^b	5 ± 3 ^b
	MS	30 ± 8 ^b	6 ± 1 ^b
	PS+MS	45 ± 18 ^{bc}	7 ± 3 ^b
II	PS	25 ± 7 ^{ab}	3 ± 2 ^{ab}
	Control	41 ± 5 ^c	13 ± 10 ^c

* average ± standard deviation from 4 replicate samples

a, b, c means that were not significantly different (p-value_t < 0.05)

Table 3.8: OTU presence or absence within each treatment. Data were collapsed for all timepoints within each treatment. A value of 1 is given for OTU presence in at least 2 replicates of that treatment. Only common OTU positions (located in ≥ 3 treatments) are shown. OTU positions are relative to each other and are based upon Molecular Analyst® Software normalization of each DGGE gel. Exp = Experiment; Treat = treatment where PS = *Phaeosphaeria spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *Phaeosphaeria spartinicola* + *Mycosphaerella* sp. 2, P+D = Particulate + Dissolved, D = Dissolved, and D+C = Dissolved + Chitin.

position →		100	108	120	129	166	178	191	200	208	216	223	226	236	253	260	269	275	282	289	299
Exp	Treat																				
I																					
	Empty	1	1	1			1	1				1						1		1	
	Natural		1			1			1				1		1	1		1		1	1
	PS	1				1			1	1		1	1	1	1			1		1	1
	MS							1		1	1	1	1	1			1	1		1	
	PS+MS	1			1	1	1				1	1	1				1	1		1	
II																					
	PS	1		1	1				1				1	1		1		1	1	1	1
	Control	1		1	1	1	1	1			1		1		1	1	1	1	1	1	
I + II																					
	P+D	1	1	1	1	1	1	1	1	1		1	1		1	1	1	1	1	1	1
	D	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	D+C	1		1	1			1	1	1	1	1	1	1	1			1	1	1	1

Table 3.9: OTU identification based upon sequencing excised DGGE bands. Classification was determined by BLAST analysis of sequence data using only matches > 90% and error values < 10^{-12} . Position = OTU positions relative to each other based upon Molecular Analyst® Software normalization of each DGGE gel.

Position	Gel	Classification
100	G	α -Proteobacteria
158	G	CFB**, Cytophaga
166	H	CFB, Flavobacteria (<i>Psychroserpens</i>)
178	F, G	γ -Proteobacteria, Burkholderiales (<i>Ralstonia</i>)
183	B	CFB, Flexibacter
191	F	γ -Proteobacteria, Burkholderia
200	D, J	CFB, Cytophaga
216	A, B	α -Proteobacteria
223	B	α -Proteobacteria, Sphingomonadales
226*	A, D, I	Chloroflexi
226*	D	CFB, Cytophaga
269	K	High G+C Gram positive, Actinomycetales (<i>Curtobacterium</i>)
275	A, G, I	α -Proteobacteria
282	H, J	α -Proteobacteria, <i>Roseobacter</i>
289	B, E, H, J	α -Proteobacteria, <i>Roseobacter</i>

* This was the only OTU to contain different sequences in bands from a similar position.

** CFB = *Cytophaga*, *Flavobacterium*, *Bacteroides*

Table 3.10: Chitinase activity for each treatment. Chitinase activity was averaged over all time points and replicates (12). PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2, P+D = particulate + dissolved, D = dissolved, D+C = dissolved + chitin.

Experiment	Treatment	Type of Leachate Addition		
		P+D	D	D+C
I	Empty	2.6 ± 1.3 ^{*,A}	2.3 ± 1.0 ^A	3.8 ± 0.8 ^A
	Natural	15.4 ± 1.2 ^{b,B}	8.0 ± 1.0 ^{a,B}	17.2 ± 5.9 ^{ab,C}
	PS	38.9 ± 9.6 ^C	20.1 ± 2.5 ^C	28.7 ± 5.0 ^D
	MS	47.7 ± 4.9 ^{CD}	37.2 ± 6.7 ^D	50.5 ± 5.8 ^E
	PS + MS	45.1 ± 9.3 ^{CD}	27.8 ± 5.4 ^{CD}	30.3 ± 7.6 ^{DE}
II	PS	33.3 ± 12.6 ^{BCD}	19.9 ± 4.3 ^C	14.3 ± 2.6 ^B
	Control	32.2 ± 9.8 ^{BC}	22.9 ± 4.5 ^{CD}	40.3 ± 11.5 ^{DE}

* average ± standard error, MUF production (nM·hour⁻¹)

^{a,b} statistically similar means within each Treatment type (within rows; p-value_t < 0.05).

All means are statistically similar for each treatment except for the Natural treatment.

^{A, B, C, D, E} statistically similar means within each Type of Leachate Addition (within columns; p-value_t < 0.05)

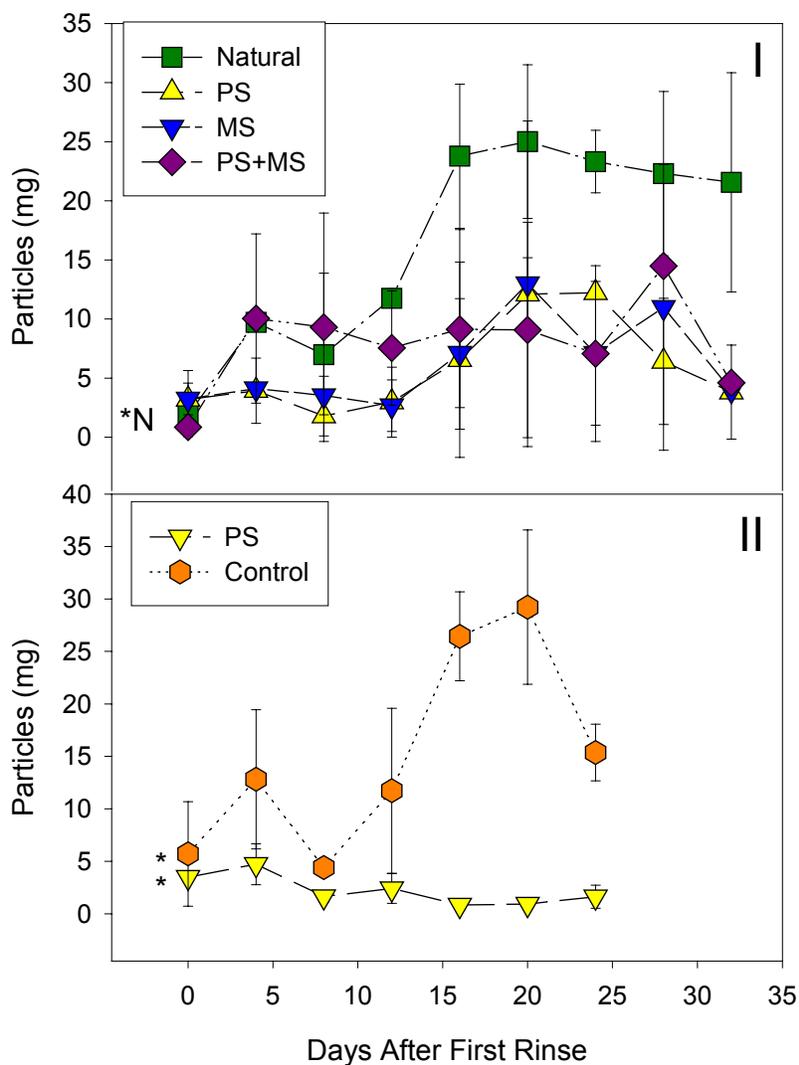


Figure 3.1: Weight of particles rinsed from microcosms. Data represent average weight change \pm standard deviation of replicate dried GF/F filters for each rinse. Data were corrected for blank values using the average value for Empty treatments (9.3 mg). (I) Experiment I results where PS = *P. spartanicola*, MS = *Mycosphaerella* sp. 2, and PS+MS = *P. spartanicola* + *Mycosphaerella* sp. 2. (II) Experiment II results. No blank was run for Experiment II, so the value from Experiment I Empty treatments (9.3 mg) were used as a blank. Asterisks (*) indicate a $p\text{-value}_{\text{zero}} \leq 0.05$ and a non-zero slope.

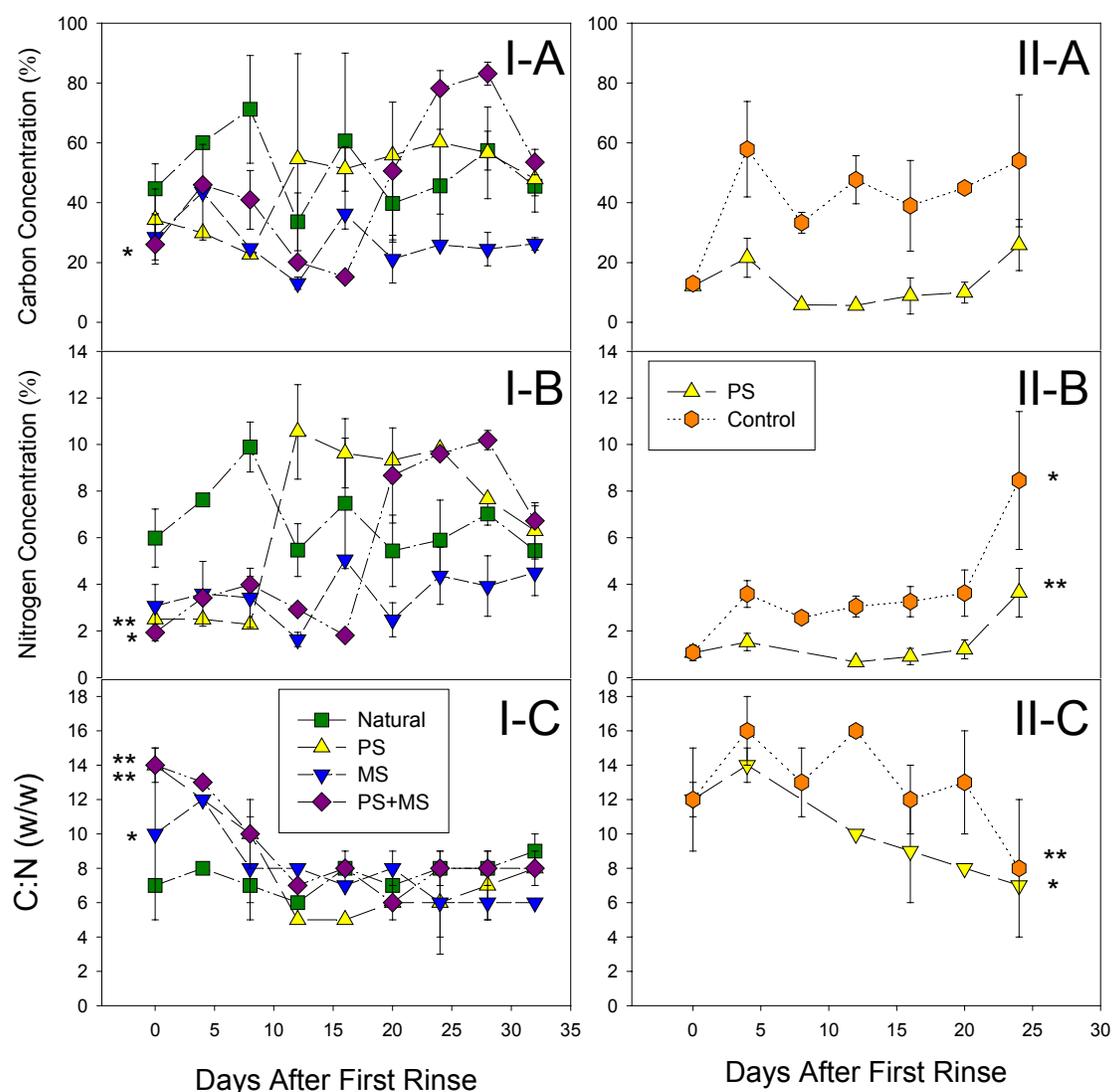


Figure 3.2: Carbon and nitrogen content of particles rinsed from microcosms. (I) Experiment 1 results where PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, and PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2. (II) Experiment II results. (A) Particulate carbon concentration was measured from replicate GF/F filters. (B) Particulate nitrogen concentration was measured from replicate GF/F filters. (C) C:N ratios were calculated from carbon:nitrogen (w/w) composition of replicate GF/F filters after correction for background carbon and nitrogen (using Empty treatment). An asterisk (*) indicates a $p\text{-value}_{\text{zero}} \leq 0.05$ and a non-zero slope. Two asterisks (**) indicate a $p\text{-value}_{\text{linear}} \leq 0.05$ and a non-linear slope. Data points represent an average \pm SD.

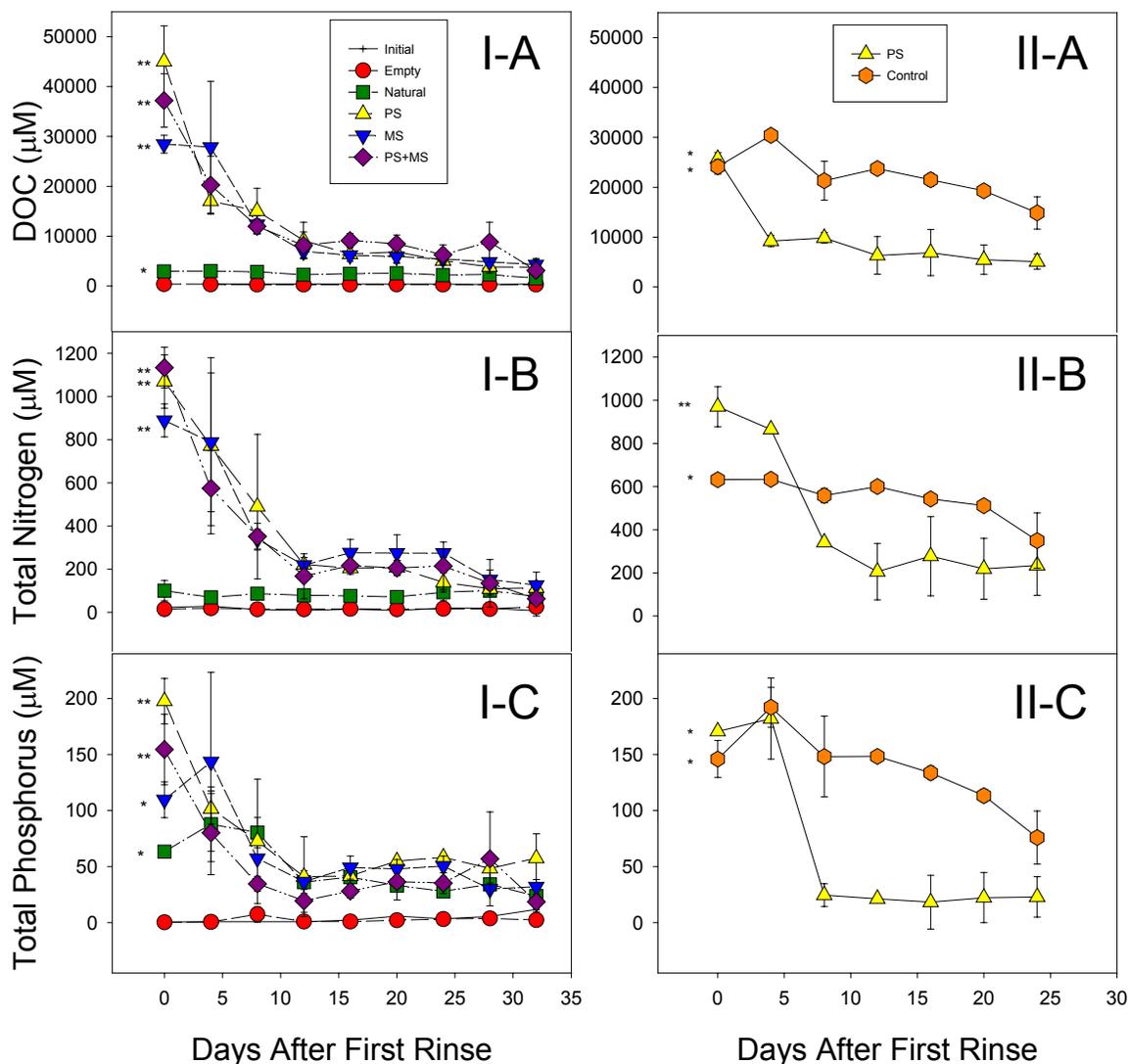


Figure 3.3: Dissolved organic carbon, total dissolved nitrogen, and total dissolved phosphate rinsed from microcosms. (I) Experiment I results where PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, and PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2. (II) Experiment II results. (A) DOC was measured from 2 replicate samples of rinse solution. (B) TN was measured from 4 replicate samples of rinse solution. (C) TP was measured from 4 replicate samples of rinse solution. (*) These treatments changed at a significant rate at $p\text{-value}_{\text{zero}} \leq 0.05$. (**) These treatments changed at a significantly non-linear rate with $p\text{-values}_{\text{linear}} \leq 0.05$. Data points represent averages \pm SD.

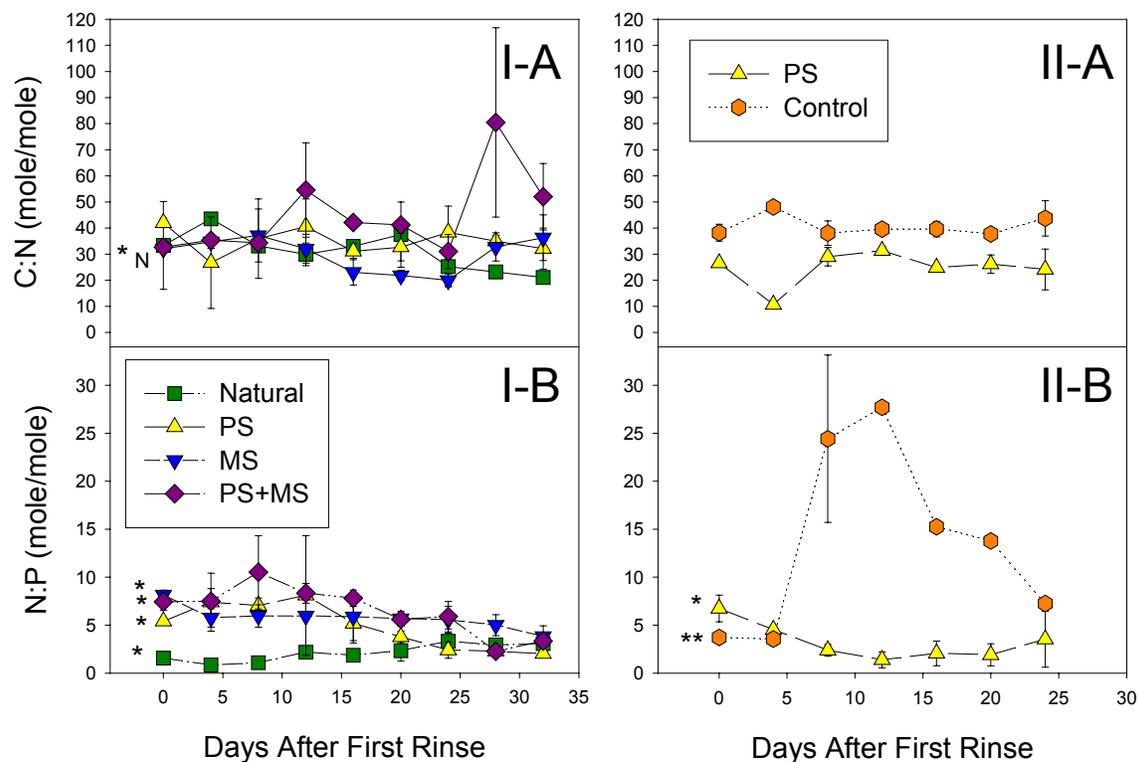


Figure 3.4: Carbon: Nitrogen and Nitrogen: Phosphorus atomic ratios of material leached from microcosms. (I) Experiment I results where PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, and PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2. (II) Experiment II results. (A) C:N was calculated using concentrations of DOC:TN (mole/mole) from 4 replicate samples of rinse solution. (B) N:P was calculated using concentrations of TN:TP (mole/mole) from 4 replicate samples of rinse solution. (*) These treatments changed at a significant rate at $p\text{-value}_{\text{zero}} < 0.05$. (**) These treatments changed at a significantly non-linear rate with $p\text{-values}_{\text{linear}} < 0.05$.

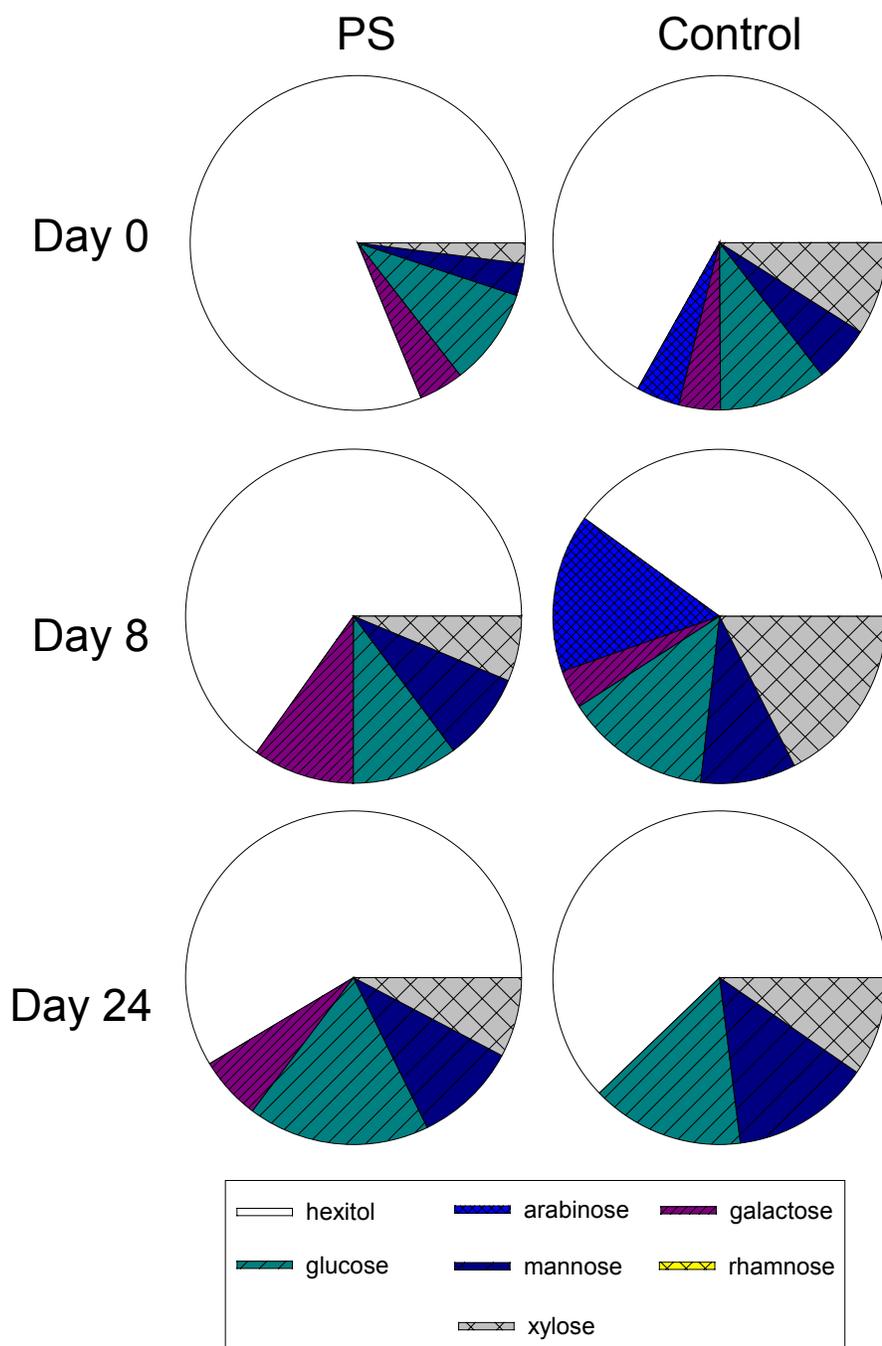


Figure 3.5: Hexitol and neutral sugars detected in leachate from Experiment II. PS = *P. spartinicola* and Control = sterile leaves. Two replicates from each time point were pooled for glycosyl analysis. Monosaccharides are shown as mole percent of total carbohydrate pool.

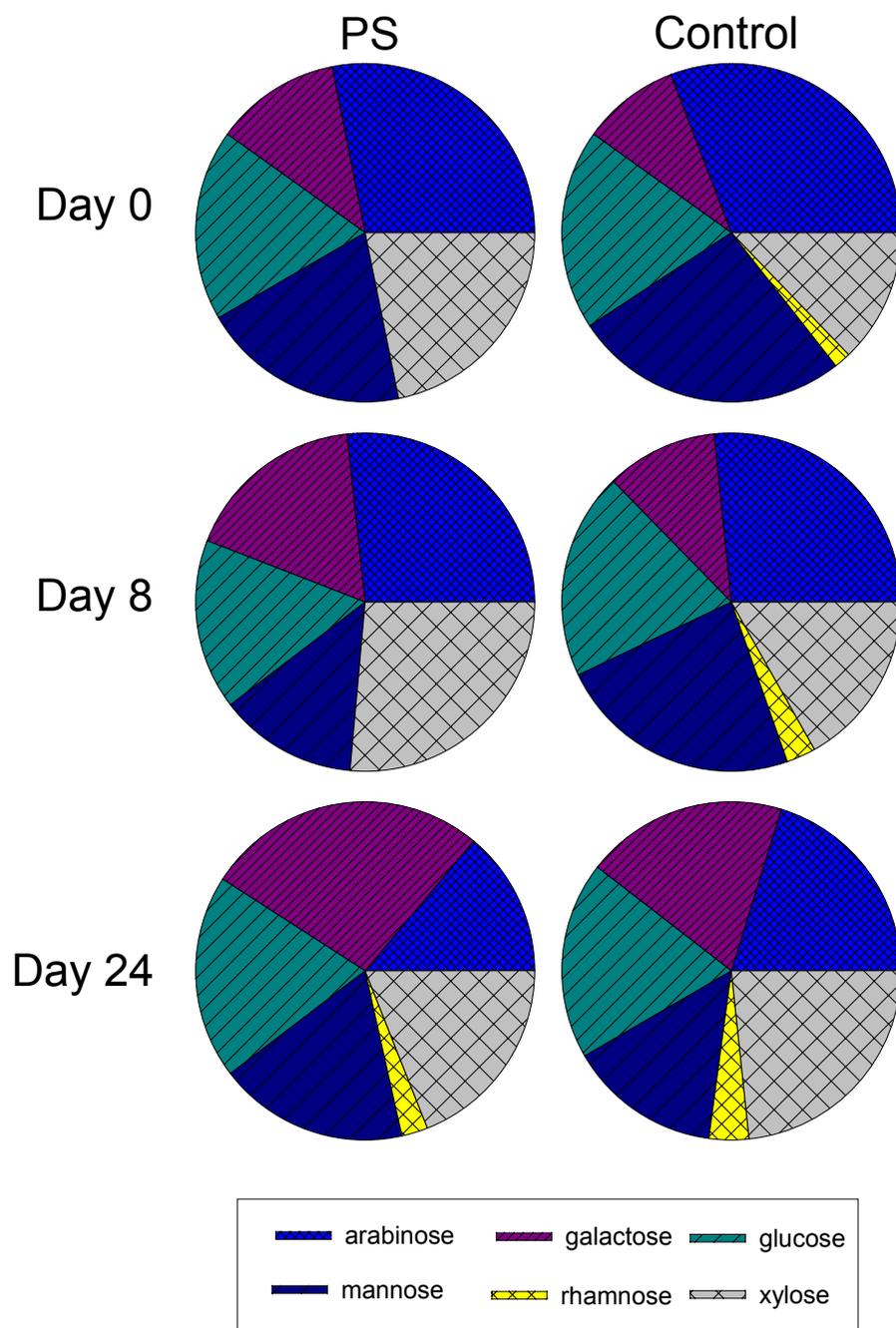


Figure 3.6: Total glycosyl composition detected in leachate from Experiment II. PS = *P. spartinicola* and Control = un-inoculated leaves. Two replicates from each time point were pooled for glycosyl analysis. Monosaccharides are shown as mole percent of total carbohydrate pool.

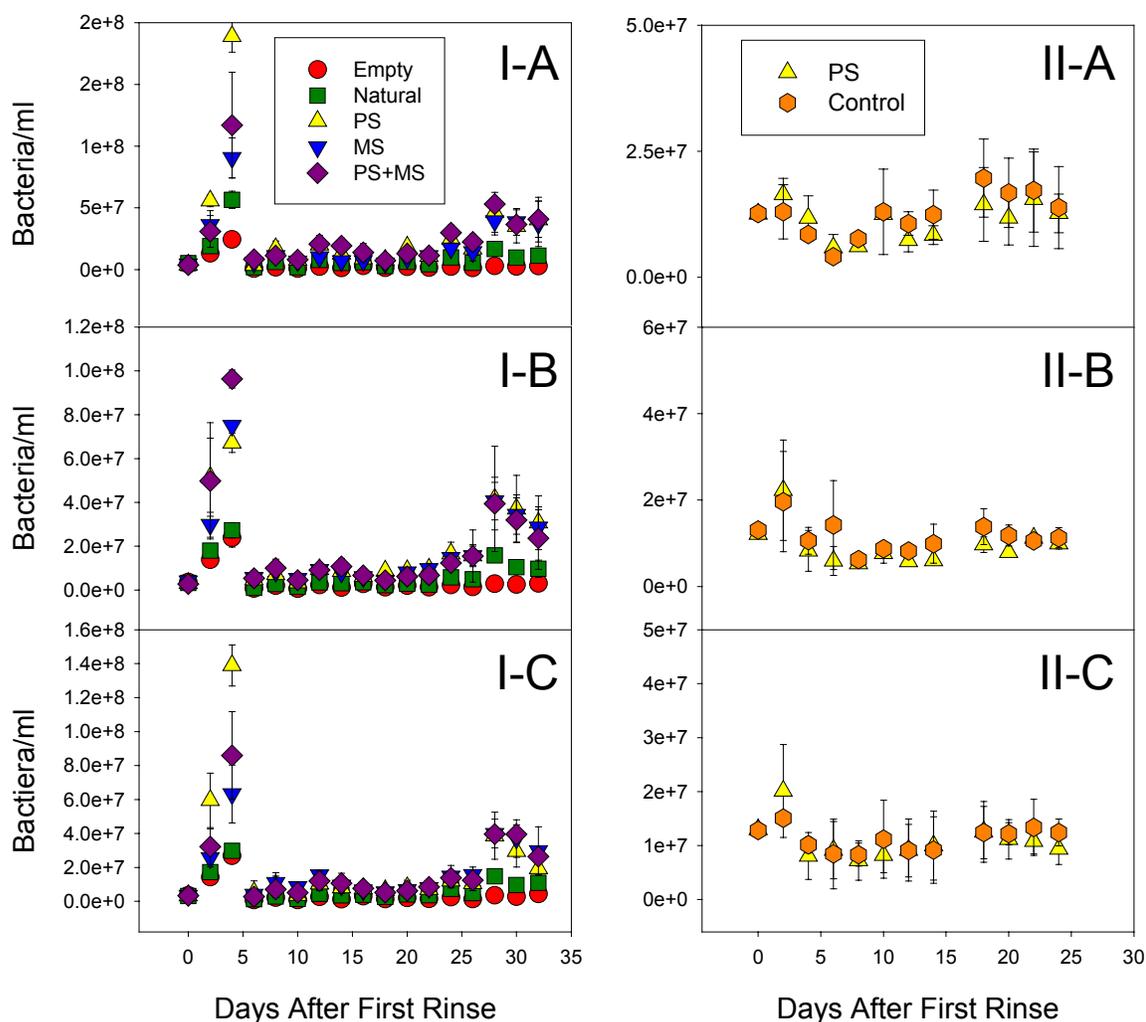


Figure 3.7: Numbers of bacteria in cultures from *S. alterniflora* leachate additions as determined by flow cytometry. (I) Experiment I results where PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, and PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2. (II) Experiment II results. (A) Counts from Particulate + Dissolved treatments. (B) Counts from Dissolved treatments. (C) Counts from Dissolved + Chitin treatments. Data points represent the average \pm SD from 4 replicate samples.

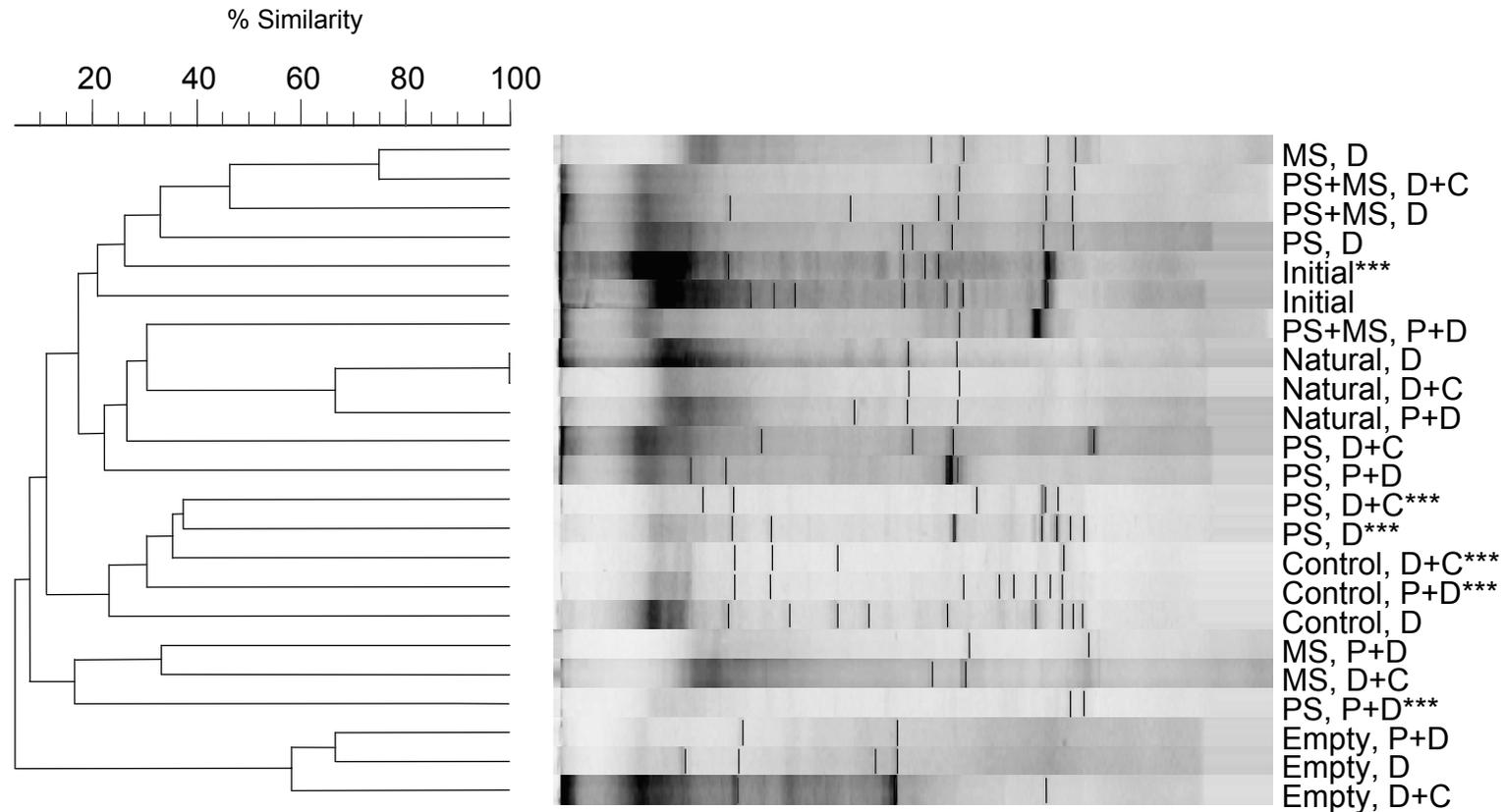


Figure 3.8: DGGE banding pattern and similarity of bacteria populations from the midpoint of each Experiment. Trees were constructed with Molecular Analyst® software using the UPGMA clustering algorithm and the Jaccard band similarity coefficient. The dendrogram scale is in % similarity of community banding pattern. PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2, P+D = particulate + dissolved additions, D = dissolved additions, and D+C = dissolved + chitin additions. (***) Denotes samples from Experiment II while remaining samples are from Experiment I. Initial samples were included for comparison.

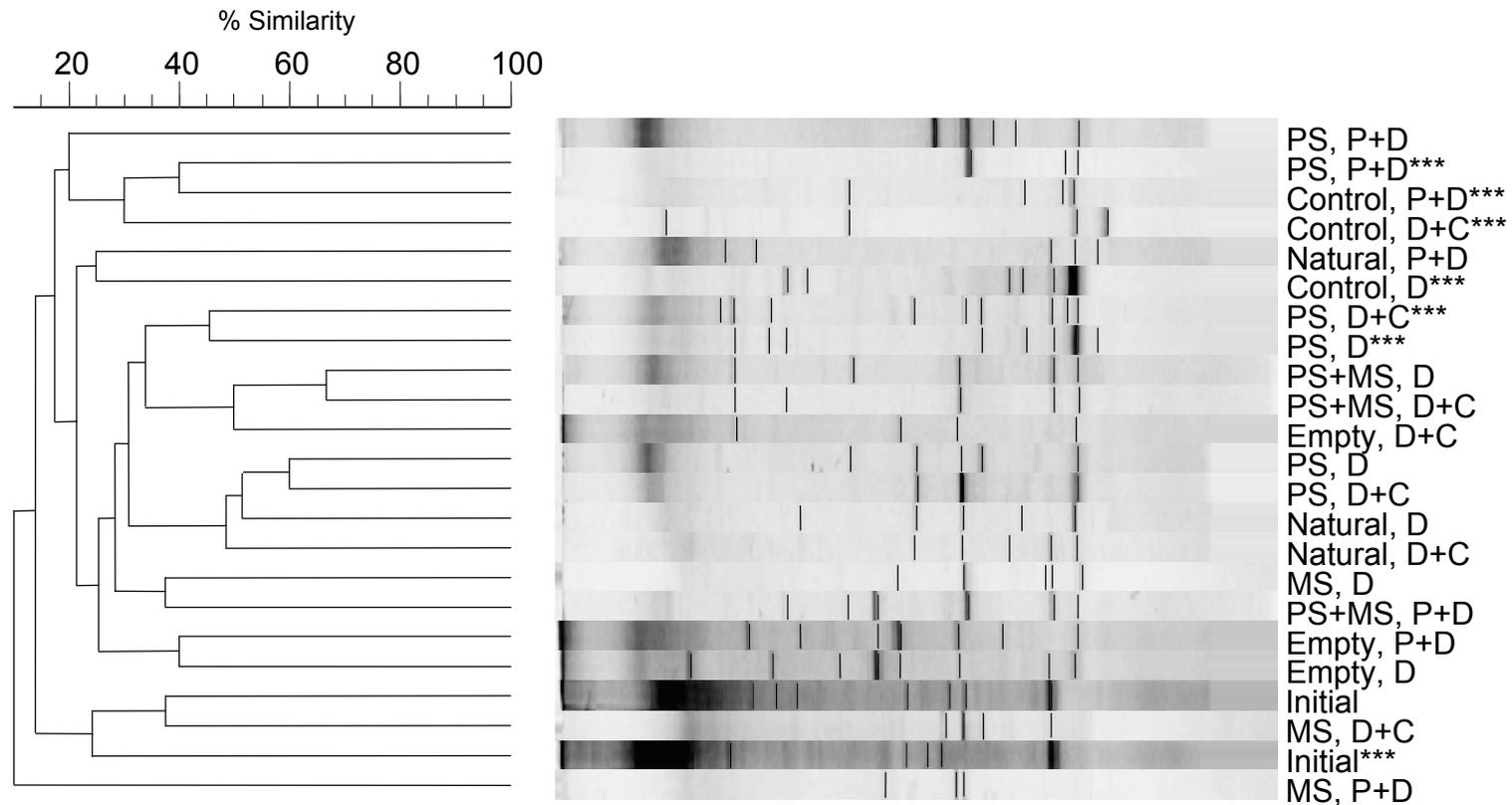


Figure 3.9: DGGE banding pattern and similarity of bacteria populations from the endpoint of each Experiment. Trees were constructed with Molecular Analyst[®] software using the UPGMA clustering algorithm and the Jaccard band similarity coefficient. The dendrogram scale is in % similarity of community banding pattern. PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2, P+D = particulate + dissolved additions, D = dissolved additions, and D+C = dissolved + chitin additions. (***) Denotes samples from Experiment II while remaining samples are from Experiment I. Initial samples were included for comparison.

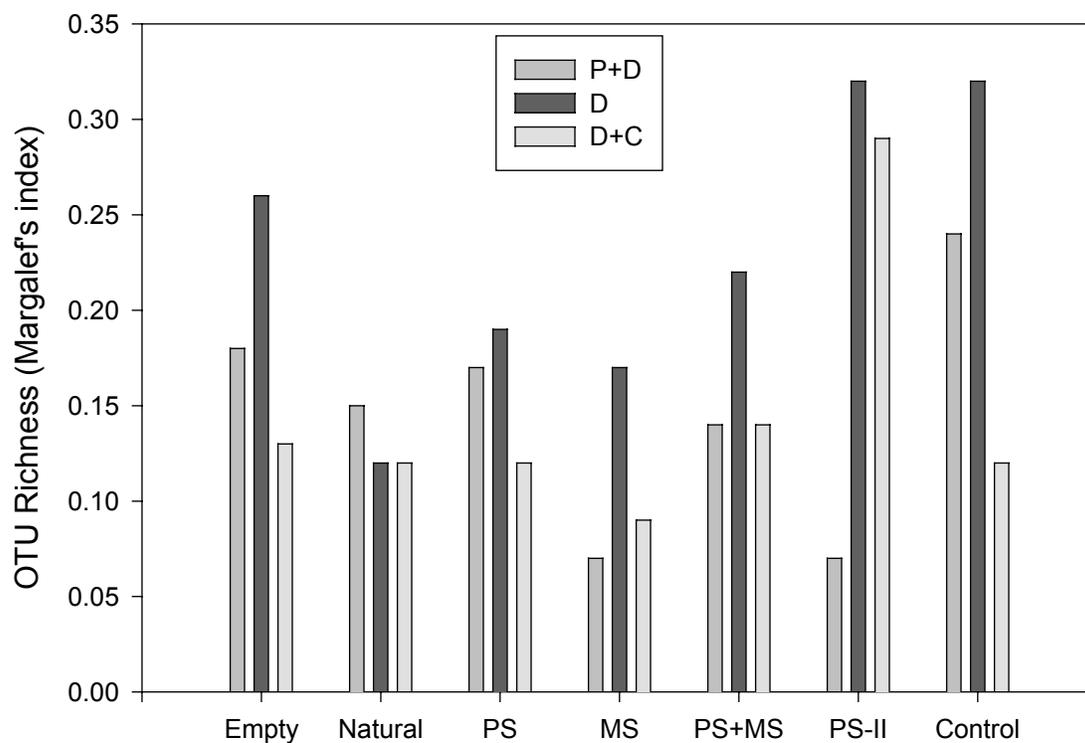


Figure 3.10: Endpoint OTU richness found in bacteria cultures receiving different leachate fractions. Average OTU richness was calculated using Margalef's diversity index based upon DGGE gel bands and bacteria counts via flow cytometry. Values are averages of two replicate treatment samples. Medium-dark bars are values from P+D treatments; dark bars are from D treatments; and light bars are values from D+C treatments. PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* and *Mycosphaerella* sp. 2, and PS-II = *P. spartinicola* from Experiment II.

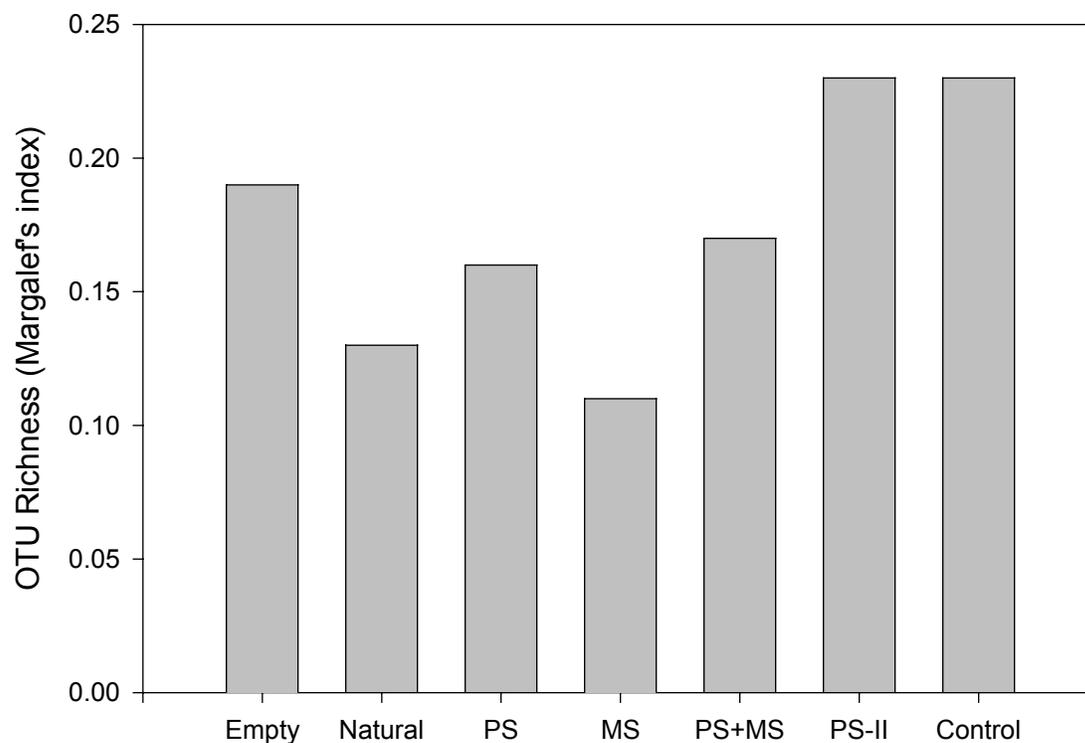


Figure 3.11: Average Endpoint OTU richness of bacteria cultures. Average OTU richness was calculated using Margalef's diversity index based upon DGGE gel bands and bacteria counts via flow cytometry. Values are averages of six replicate samples. PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* and *Mycosphaerella* sp. 2, and PS-II = *P. spartinicola* from Experiment II.

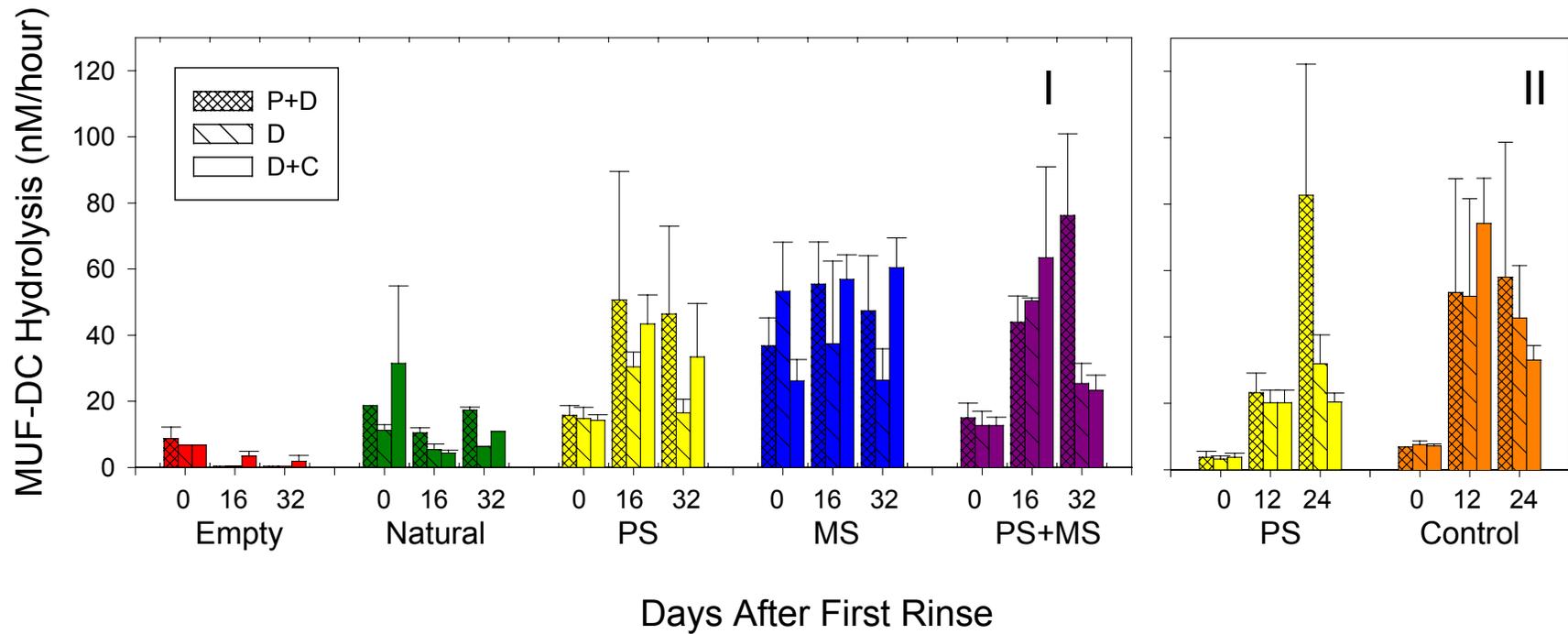


Figure 3.12: Chitinase activity in bacteria cultures as measured from 4 replicate MUF-DC incubations at 3 time points. (I) Experiment 1 results where PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, and PS+MS = *P. spartinicola* + *Mycosphaerella* sp. 2. (II) Experiment II results. P+D, D, and D+C treatments are shown separately where P+D bars are crisscrossed, D bars are striped, and D+C bars are solid. Data are averages \pm SD from 4 replicate MUF-DC incubations.

CHAPTER 4

DISCUSSION

I. Summary

- A. Does the presence of ascomycetes modify decaying *Spartina* leaves and leachate?

Regardless of the fungal community, the chemical properties of cut, standing dead *S. alterniflora* blades did not change substantially over 29-39 days. Specifically, neither leaf organic density nor leaf nitrogen concentration changed, while only minimal increase in ash content and decrease in carbon concentration was observed. The presence of ascomycetes affected leaf carbon content because treatments containing fungi (Natural, PS, MS, PS+MS) showed significant decreases in and/or significantly lower concentrations of leaf carbon.

More particles were measured in leachate from decaying *S. alterniflora* when fungi were absent. The C:N ratio of particles rinsed from leaves decreased during these experiments, but the C:N ratio of the particles was independent of the species of fungus. In addition, the concentrations of dissolved carbon (DOC), nitrogen (TN), and phosphorus (TP) leached from leaves decreased exponentially with time in treatments containing fungi. Without fungal growth, DOC, TN, and TP concentrations in leachate decreased linearly.

Therefore, based upon the results of these experiments, it appears that *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. 2 may not greatly influence the chemical properties of decaying *Spartina* leaves, but they do modify the properties of *Spartina* leachate. Specifically, living *P. spartinicola* appears to prevent fragmentation of leaves. Together or alone, *P. spartinicola* and *Mycosphaerella* sp. 2 also reduced leaching of DOC, TN, and TP from *Spartina*.

B. How are bacteria affected by fungally-modified leachate added as a growth substrate?

While the amount of cultured bacteria fluctuated during these experiments, the type of leachate we added did not affect bacterial production. Therefore, neither the identity of ascomycete (*P. spartinicola*, *Mycosphaerella* sp. 2, or both) nor the fraction of leachate (particulate + dissolved, dissolved) generated during *S. alterniflora* decomposition influenced the production of bacteria biomass when ascomycetes were physically separated from bacteria. Bacterial community composition changed in response to leachate additions, and the major taxa we found are also found associated with *S. alterniflora* leaves in nature. In order of decreasing abundance of identified OTUs, α -Proteobacteria, *Cytophaga-Flavobacteria-Bacteroides* (CFB), γ -Proteobacteria, and Gram-positive bacteria were all detected. OTU richness (Margaleff's index) was higher when the bacteria were given just the dissolved portion of the leachate as added substrate, but it appears that in general, fungally-modified leachate does not strongly affect OTU richness within bacteria populations growing on leachate.

- C. Could bacterial chitinase activity play a role in interactions between fungi and bacteria in the *Spartina* decay system?

Bacteria did not respond by extra growth in response to chitin amendments; therefore, bacteria from the *Spartina* decay system may not be conditioned to use chitin as a growth substrate. This suggests that since fungi contain chitinous cell walls, the bacterial population may not be conditioned to use fungi as a growth substrate as well. In addition, OTU richness was generally lower when chitin was added to the cultures suggesting selection for favored OTUs. Since bacterial abundance did not change, lower OTU richness suggests that bacterial communities become more specialized to use chitin for a substrate. If this is the case, then as we hypothesized, chitinase activity may be increased in chitin-enriched treatments. However, not only did chitinase activities decrease in chitin-enriched treatments, chitinase activity was generally lower in treatments grown with leachates from fungally-modified leaves. This may indicate that MUF-DC is not a good indicator of chitinase activity under these experimental conditions. However, all of these conclusions imply that chitinase activity may not play an important role in fungal/bacterial interactions.

These results are discussed in more detail below and are compared with the literature. We discuss potential flaws in the design of the experiments while presenting possible solutions. Lastly, we propose ideas for how these results can better explain the interactions between fungi and bacteria as they degrade detritus from the common salt marsh plant, *S. alterniflora*.

II. Leaves Inoculated with Fungi

A. Fungal Growth and Community Composition

The ergosterol values I measured (422 – 923 $\mu\text{g} \cdot \text{g}^{-1}$ leaf organic matter (OM) from Experiment I; 233 – 1056 $\mu\text{g} \cdot \text{g}^{-1}$ OM from Experiment II) are within the range found on blades of standing dead *S. alterniflora* at sites from Maine to Florida (337 ± 95 to 714 ± 339 $\mu\text{g} \cdot \text{g}^{-1}$ OM; Newell et al. 2000). By the end of each experiment, however, ergosterol content was higher in treatments that were inoculated with fungi than has been reported in the literature. This is expected because experimental conditions were controlled to promote optimal fungal growth without grazers.

Even though hyphae were visible throughout Experiment I, no significant increase of ergosterol was measured, suggesting that fungi did not grow. One possibility for this discrepancy is that ergosterol values for Experiment I were underestimated because some of the samples spilled during transport. In addition, few replicate samples were collected for analysis. For example, only one sample (3 pooled leaves) was taken from each treatment at the midpoint of the experiment. However, the lack of ergosterol increase in the fungal treatments in Experiment I may just reflect 'old' ergosterol converting into new hyphal walls.

To address this problem in Experiment II, triplicate samples were taken at each time point. In addition, extra effort ensured that samples were not compromised during transport. Ergosterol data from Experiment II were more consistent and showed significant growth of *P. spartinicola* ($26 \mu\text{g} \cdot \text{g}^{-1}$ OM $\cdot\text{day}^{-1}$; $p\text{-value}_{\text{zero}} < 0.001$). Ergosterol quantities better reflected observed hyphal growth in this experiment. In confirmation of adequate growth conditions, ascospores were visible on the leaves of

the *P. spartinicola* treatment (PS) at the end of the experiment (29 days after inoculation). No fungal growth was detected (visibly or by ergosterol content) in the Control, nor were ascospores noted on Control leaves at the end of the experiment.

The presence of inoculated fungi within the leaves was confirmed by TRFLP. In the Natural treatment, we identified *P. spartinicola*, *Mycosphaerella* sp. 2, *P. halima*, and *Buergenerula spartinae* and '4clt' using TRFLP. In nature, the same ascomycetes dominate fungal populations (Newell et al. 2000; Buchan et al. 2002).

Due to contamination with *P. halima*, data from the Control in Experiment I could not be used as intended. In this treatment, aerial hyphae were visible in both replicate microcosms after 10 days of incubation. TRFLP data suggest that this contaminant was *P. halima*. It is possible that this ascomycete was not fully eradicated from the *Spartina* leaves by autoclaving, but it was not detected in the other treatments by TRFLP. Gamma radiation did not kill all of the fungi either. However, the possibility of incomplete sterilization led me to use more stringent and thorough sterilization in Experiment II.

B. Leaf Decomposition Measures

Since the total dry weight of the leaves was not measured after each experiment (due to subsampling), the total decomposition of leaves could not be quantified. Similarly, total weights of carbon and nitrogen loss could not be calculated. Instead, leaf properties such as organic density, ash content, carbon, and nitrogen were measured as percentages of leaf dry weight or area. Therefore, the changes in leaf quality over time and between treatments are relative measures of lability.

Organic density (5.2 – 13.3 mg · cm⁻² of leaf for Experiment I; 4.7 – 12.8 mg · cm⁻² of leaf for Experiment II) was within the range found on blades of standing dead *S. alterniflora* at sites from Maine to Florida (6.0 – 14.2 mg · cm⁻² of leaf; Newell et al. 2000). Over time, leaf organic density appeared to decrease in each treatment and Experiment, but this decrease in density was not significant ($p\text{-value}_{\text{zero}} > 0.05$). It is likely that this decrease in organic density was real, but the lack of sample replication did not compensate for noise in the data. This is consistent with other studies reporting seasonal decreases in organic density (e.g. Newell et al. 1989) and those reporting decreased organic density in later decay stages (Buchan et al. 2003).

Percent ash values for standing dead *S. alterniflora* (17 - 41%) were consistent with those previously reported by Valiela et al. (1985) and slightly higher than reported by Buchan et al. (2003) and Burkholder (1956). If these leaves had been collected from the sediment surface and not rinsed before use in these experiments, ash content would have been higher (Valiela et al. 1985).

Carbon content found in these experiments (30 - 40 % of leaf material) is also consistent with reported values for *S. alterniflora* (Valiela et al. 1985; White and Howes 1994a). In the literature, however, carbon concentrations vary little over long time scales. For example, Valiela et al. (1985) found that *S. alterniflora* leaf carbon decreased by $\sim 0.06\% \cdot \text{day}^{-1}$ during 600 days of incubation in litterbags in Great Sippewissitt Marsh, New England. In the present study, leaf carbon concentration declined at a significant rate ($0.1 - 0.2\% \cdot \text{day}^{-1}$) in all treatments over 40 days except for the Control in Experiment II. The higher rate of decline in these experiments could be caused by several factors attributable to the experimental design, including

autoclaving, constant moisture, aeration and protection of fungi from grazing. Also, my experiments were conducted for < 40 days, so rates may reflect shorter-term changes in the more labile components of leaves. However, it is probable that the rate of leaf carbon decrease I measured may not be significantly different from that measured by Valiela et al. (1985).

Of all the leaf properties, carbon content was one of three to demonstrate a treatment effect. All treatments except the Control in Experiment II exhibited a loss of leaf carbon. Since those treatments all incorporate biological degradation, this suggests that microbial processes can mobilize leaf carbon at faster rates than abiotic processes. In addition, carbon content decreased much faster when *Mycosphaerella* sp. 2 was present alone (MS) than in any of the other Fungal treatments (PS, PS+MS). The data for that treatment were highly variable, however, so even though this difference in rates is significant, it may not be reproducible.

Nitrogen content (0.5 – 0.7 % of leaf material) was similar to reported values (0.38 – 2 %) (Gallagher et al. 1980; Hopkinson and Schubauer 1984; Valiela et al. 1985; White and Howes 1994a). Nitrogen content did not change significantly over 40 days in any treatment. In nature, however, leaf nitrogen content increases slowly over long periods of time (Godshalk and Wetzel 1978b; Rice and Tenore 1981; Valiela et al. 1985; White and Howes 1994a). It is likely that the short duration of my experiments did not allow adequate time this trend to be detectable.

Nitrogen content was the second of three leaf properties to show a treatment effect. Although there was no significant change in nitrogen content during the

experiment, leaf nitrogen content was significantly lower on average in PS+MS than in the Natural treatment.

Calculated C:N (mass) ratios (49 - 80 for Experiment I; 60 - 68 for Experiment II) are within the range of values found in standing dead *S. alterniflora* leaves at sites from Maine to Florida (32 - 74) taken from Newell et al. (2000). Leaf C:N ratios did not change significantly over the time period of these experiments. This slow change is consistent with slow microbial decay (Valiela et al. 1985).

C:N was the third leaf property that showed a treatment effect. On average, C:N of leaves in the Natural treatment was lower than in the MS and PS+MS treatments. This is primarily a factor of higher nitrogen concentrations in leaves from the Natural treatment. Elevated nitrogen loss (as dissolved TN) from Fungal treatments in comparison to the Natural treatment may reflect the effect of autoclaving on nitrogen mobility. Because Controls were contaminated during Experiment I, we cannot determine whether fungal activity or autoclaving was the dominant factor affecting the leaf C:N ratios.

In general, the properties measured suggest that the leaves we used are in the late leaching stage to early microbially dominated stage of decomposition (Valiela et al. 1985). Leaves were collected as standing dead—already brown—which suggests that they were significantly colonized by fungi and that substantial leaching has already occurred. The standing dead blades used in Experiment II were from the same growing season as those used in Experiment I. The only difference was that leaves used in Experiment II had remained in the marsh longer (November through May), allowing them to decompose further before we used them. Therefore, the nutrient content of

leaves used in Experiment II should be equal to or lower than in leaves used for Experiment I. While carbon content (per dry weight) was similar in each experiment, nitrogen content of the leaves used in Experiment II had increased relative to those used in Experiment I. Increased nitrogen content of decaying leaves is well-documented, with low % N in the summer/fall and higher % N in the winter/spring. The increased % N may be due to nitrogen-enriching processes such as N-fixation or nitrogen-binding phenolics (Wilson et al. 1986). The increased nitrogen content can be also be seen in the lower C:N ratios of leaves in Experiment II compared to Experiment I. This temporal decrease in C:N is also found in nature (Valiela et al. 1985; Newell 2001b)

III. Particulate Material Released from Decaying Leaves

A. Particle Quantity

The weight of particles rinsed from the microcosms varied between treatments. On average, similar quantities of particles were removed from Fungal treatments (PS, MS, PS+MS) in Experiment I at each rinse (~5 mg). Significantly more particles were rinsed from the Natural treatment. Because leaves in the Natural treatment were not autoclaved, one might expect fewer particles to be lost, since leaf integrity was not compromised. However, the lack of autoclaving allowed other biota such as insects, herbivores, or other microbes to grow. Spiders were seen in Natural treatments during preliminary experiments while two flies were noted in Experiment I. The presence and activity of these macro-organisms may help to explain the increased particles collected from these treatments.

Significantly fewer particles were lost on average when *P. spartinicola* was present (Experiment II). Considering that *P. spartinicola* physically penetrates *S. alterniflora* tissues during decomposition (Newell et al. 1996), one might expect that cellular disruption would decrease plant material integrity, therefore generating particles. However, my data suggest that *P. spartinicola* may retard loss of particulate material from *Spartina* leaves.

The amount of carbon and nitrogen lost as particulate material from the microcosms increased over time in most of the treatments. In Experiment I, the average amount of particulate carbon and nitrogen lost from each microcosm followed this pattern: Natural \geq PS+MS \geq PS > MS > Empty. In Experiment II, particulate carbon and nitrogen loss were significantly higher in the Controls ($p\text{-value}_t < 0.01$).

As a note, the amount of particulate material available for carbon and nitrogen analyses was low. Even though replicates were fairly consistent and standards were precise (Coefficient of variance < 4.0), the amount of material may have been at or below the detection level for the CHN analyzer in some cases.

B. Particle Carbon and Nitrogen

Different treatments generated variable amounts of particulate material at each rinse, but in order to assess the relative quality of the particles, concentrations of carbon and nitrogen content were determined. The carbon content of the particles was variable (~20 - 80%; w/w). Compared to carbon content of the leaves, particles from Fungal treatments were initially depleted in carbon (Figure 4.1.A). Carbon was also depleted in the particles relative to the leaves during Experiment II. These findings are similar to those of Greenway (1994), who demonstrated that carbon content of litter decreased

with decreasing particle size. If the particulate material was separated from the leaves by physical/abiotic forces, leaves and the particulate material collected on the same day likely have the same carbon content. However, if particles have different carbon content than leaves, it is possible that a biological process was involved. Some evidence for this may be found by reviewing transmission electron microscopy (TEM) pictures of *S. alterniflora* material pervaded by *P. spartinicola* (Newell et al. 1996). In these TEM pictures, “granular or amorphous darkened digesta of fiber-cell material” can be seen outlining cavities of digested plant material surrounding fungal hyphae. Assuming this granular digesta is fine particulate material that can be rinsed from the leaves, it easily follows that biological processes can alter carbon concentrations of particles relative to leaves.

The nitrogen content of particles was also variable (0.4 – 10 %; w/w). With only one exception (midpoint, PS, Experiment II), nitrogen was enriched in the particles relative to the leaves (Figure 4.1.B). Particles were enriched more during Experiment I. These results are consistent with Greenway (1994) who demonstrated that nitrogen content increased with decreasing particle size of litter. Again, since the particles generally had more nitrogen than the leaves, it is possible that some biotic process or incorporation of a nitrogen-rich component, such as fungal cell walls, affected the composition of particulate material.

On average, particle C:N was higher in Experiment II than in Experiment I. As discussed above, this could reflect leaf collection date, for leaves used in Experiment II had more time to decompose in the marsh before use. Since the two experiments were

run for different lengths of time, however, the difference may also be due to prolonged degradation.

In Experiment I, average particle C:N was higher in all leaf treatments (Natural, PS, MS, PS+MS, Control) than in the rinse solution (Empty). This reflects the addition of particles derived from decaying leaves (higher in carbon) to the material collected on GF/F filters. None of the Fungal treatments (PS, MS, PS+MS) produced significantly different particle C:N, suggesting that *P. spartinicola* and *Mycosphaerella* sp. 2, alone or in combination, do not influence particle C:N.

In Experiment II, particulate C:N decreased over time in a similar fashion for PS and the Control. These results suggest that increasing nitrogen enrichment of the particles is not due to fungal pervasion since no active fungal growth was detected in the Control. Instead, it is likely that particulate C:N decreased because of abiotic properties of leaching.

Relative to the leaves, particle C:N is lower (Table 4.1). This indicates that particulate material lost from leaves is depleted in plant carbon compared to nitrogen or that nitrogen is enriched. This is consistent with the findings of Greenway (1994) who demonstrated that plant litter C:N decreased with decreasing particle size. The difference in C:N between leaves and particles may also be due to error in detection levels because small quantities of particles were used for measurement.

IV. Leachate from Decaying Leaves

A. DOC, TN, and TP Concentrations

In order to compare DOC, TN, and TP concentrations in leachates from my experiments with those found in other *Spartina alterniflora* leachates, the concentrations

had to be converted to rates of carbon, nitrogen, and phosphorus leached per gram of leaf DW per hour. To accomplish this, several assumptions were made and tested. First, since the total dry weight of leaves was not measured during these experiments, it was assumed that 22 g DW of material was in each microcosm. This assumption was tested and validated by taking 25 g (wet weight) of leaves, drying them, and achieving an average weight of ~22 g. A second assumption is that the leaf DW did not change significantly during the course of these experiments. No statistically significant difference in average dry weights of leaf subsamples was found, so this assumption was also validated. Third, unlike other studies, these leaves were not submerged; therefore, I assumed leaves were saturated for 15 minutes during the rinsing period. This assumption was not tested; rather, it is likely an overestimate of submersion time. Therefore, calculated rates of DOC, TN, and TP leached from these experiments are likely underestimates of actual rates.

Table 4.2 shows literature values of DOC leaching rates for comparison with those from the present study. Rates of DOC release in the Natural treatment are comparable to previous findings. However, in every other treatment, DOC was leached at rates ≥ 10 times higher than previously reported, even though the estimates from these experiments err toward underestimation. Several explanations for the comparatively high rates of DOC release are possible.

First, many of the previous studies (Turner 1978; Turner 1993) calculated leaching rates from green (live) leaves still attached to the plant. Hopkinson and Schubauer (1984) have demonstrated that living *S. alterniflora* can translocate 54% of photosynthate to rhizomes for storage. This decreases the amount of material available

to be leached from living leaves. In contrast, leaves cut from plants cannot redistribute nutrients to other plant locations thus potentially increasing leachable nutrients.

However, since the leaves I cut were already dead, this explanation is not likely.

Second, previous studies recorded leaching rates averaged after a 2 – 3 hour incubation (Gallagher et al. 1976; Turner 1978; Hopkinson and Schubauer 1984; Pakulski 1986; Turner 1993). However, leaching rates are not constant over that time scale. For example, Turner's (1978) results indicate that carbon is initially lost at higher rates during the first 40 minutes of submergence. Similarly, Gallagher et al. (1976) found lower rates of leaching in longer incubations, but they concluded that decreased rates were due to microbial activity. Therefore, the longer the incubation, the lower the average leaching rate.

Third, due to the initially heightened and variable leaching rates, Turner's studies (Turner 1978; 1993) included a 30 minute soak before collecting leachate from the leaves. Since those studies focused on long-term leaching rates, these incubations were necessary. However, that also led to underestimation of total loss, for this initial leaching removed material that had accumulated on the leaves since the previous wetting episode. Not including this material in the accumulated leachate underestimated the total loss of nutrients in Turner's studies and therefore decreased estimated leaching rates.

Forth, the present study is unique in experimental design because leaves were autoclaved. By comparing DOC released from autoclaved versus non-autoclaved leaves, it is clear that autoclaving greatly increases DOC loss as leachate. However, the relative amounts of carbon and nitrogen (C:N) leached are not significantly altered

by autoclaving. This suggests that, although DOC and TN loss increase when the leaves are autoclaved, they are released proportionately.

Even though leaching rates from the current study are 10 times larger than previously recorded, comparisons between treatments and changes over time are useful. This study is unique because microbes were intentionally added to the leaves. Past studies either ignored epiphytic microbial activity as it related to leaching rates or they controlled minimally for it by rinsing and gently wiping leaves before incubation. In this study, average DOC loss from leaves is significantly higher (> 2 times; $p\text{-value}_t < 0.001$) in the absence of fungi. Therefore, presence of fungi and/or their activity decreased rates of DOC loss from leaves.

DOC, TN, and TP rinsed from the leaves decreased exponentially in the Fungal treatments. The initially elevated concentration of these substances in the rinse solution may result from autoclaving and by the accumulation of leachable material between rinses. In fact, there is a large difference when comparing loss of dissolved material between autoclaved and non-autoclaved leaf treatments (Natural versus PS, MS, PS+MS, Control). DOC and TN concentrations in leachate are lower and more constant in the Natural treatment, while Fungal treatments produced much higher DOC and TN concentrations in leachate that declined exponentially. In addition, by the end of Experiment I, the concentrations of material in leachates from autoclaved treatments had declined to levels comparable with the non-autoclaved treatment.

This does not necessarily mean that autoclaving causes concentrations of dissolved nutrients to decline exponentially in leachate. As evidence, both treatments in Experiment II contained autoclaved leaves. Leachates from the Control initially

contained elevated concentrations of dissolved material, but these concentrations declined linearly, not exponentially as seen with Fungal treatments. In fact, these results suggest that, while autoclaving greatly increases the initial leachability of plant carbon, it is the presence of fungi that caused the exponential decrease of the released dissolved material.

Could lower concentrations of material in leachates from the Natural treatment be caused by factors other than autoclaving? The Natural treatment was designed to observe how natural populations of fungi, bacteria, and other epiphytes affect leaching properties. Bacteria are known to use *S. alterniflora*-generated DOC directly with high growth efficiency (Findlay et al. 1986; Hullar et al. 1996). The presence of bacteria in addition to fungi could explain lower DOC release from Natural treatment leaves. This explanation is reasonable, but my data cannot demonstrate it unambiguously due to the issues of autoclaving discussed above.

Nitrogen and phosphorus release followed similar patterns to DOC; however, a few important differences were noted. In Experiment II, the PS treatment initially released more TN than the Control. This could be due to release of accumulated highly nitrogenous compounds, such as lignocellulytic enzymes, by fungi. Whatever the reason, it appears to be biological. However, six days after the first rinse, TN concentration in PS leachates fell below the Control.

Examining the results from Experiment II, a tentative order of immobilization for carbon, nitrogen, and phosphorus is suggested. When *P. spartinicola* was present, the first material to be immobilized from leachates was DOC (day 4) followed by TP (day 8) and TN (day 12). Even though these materials were depleted, they were by no means

exhausted. Assuming that the exponential decrease of DOC, TN, and TP rinsed from PS was a result of fungal uptake, it is possible that of the dissolved materials, carbon was limiting first, phosphorus second, and nitrogen third.

B. Stoichiometry of Leached Carbon, Nitrogen, and Phosphorus

Atomic C:N of dissolved material only changed significantly over time in the Natural treatment leachate. This indicates that carbon and nitrogen are leached at similar rates. C:N values were similar between experiments, having a large range but averaging 34 ± 20 . In addition, the average dissolved C:N from each treatment was similar to that from the rinse solution (Empty treatment; $p\text{-value}_t > 0.05$). This suggests that carbon and nitrogen were not affected. Since the rinse solution was collected from the environment, it also suggests that ambient C:N in the water column is similar to C:N of material released as leachate from *S. alterniflora* leaves.

The average C:N (atomic) ratio was ~ 2 times higher in the leaves than in the leachate (Table 4.1). This suggests that nitrogen is enriched in the rinse solution, a result also found by Turner (1993). However, average C:N of dissolved material was higher than in the fine particulate matter, suggesting further nitrogen enrichment in particles relative to leachate.

In contrast, average atomic N:P of material in leachate from each leaf treatment (Natural, PS, MS, PS+MS) was significantly lower than that of the rinse solution (Empty treatment; $p\text{-value}_t < 0.05$). This suggests that leached phosphorus was comparatively enriched. Average leachate N:P was similar between Fungal treatments (average = 6 ± 2 ; $p\text{-value}_t > 0.05$) but Natural leachate N:P was significantly lower (average = 2 ± 1 ; $p\text{-value}_t < 0.001$). Again, due to autoclaving, it cannot be said that N:P is lowered when

full microbial assemblages are present (Natural treatment). It is possible, then, that phosphorus in the leachate is elevated in comparison to nitrogen due to fungal activity and leaf decomposition, but autoclaving complicates interpretation of this effect.

C. Dissolved Carbohydrates

Sugar alcohols were the most predominant form of glycosyl residues found dissolved in the leachate, regardless of treatment. However, sugar alcohols make up a higher mole percentage of the material dissolved in leachate from PS treatments. Acyclic sugar alcohols have been shown to be storage carbohydrates and osmolytes of fungi during water stress (Brown 1990; Blomberg and Adler 1992; Money 1994; Jennings 1995), so it is not surprising to find more sugar alcohols present in leachate from the PS treatments. Of the neutral sugars detected, galactose, glucose, mannose, and xylose were present in PS treatment at all sampling times. Those four sugars were present in the Control, and arabinose was also present in the first two time points of the Control.

When comparing total glycosyl composition, mannose, glucose, galactose, xylose, and arabinose were present in all treatments and time points sampled. Each of these carbohydrates was also present in similar molar percentage. Because arabinose as a neutral sugar was only detected in low quantity, this suggests that the arabinose present in leachate is mostly bound in sugar alcohols. In a study by Jennings (1995), non-growing mycelia of *Dendryphiella salina* produced mannitol and arabitol in response to increased salinity. Arabinose is the carbohydrate component in the sugar alcohol arabitol, so my findings suggest that, if *P. spartinicola* osmoregulates in a fashion similar to *D. salina*, *P. spartinicola* produced arabitol in response to the

increased salinity of the rinse solution. In addition, low mole percentages of another glycosyl residue, rhamnose, were detected in the total-carbohydrate fraction. This suggests that rhamnose, too, is present as a sugar alcohol. It is not clear why rhamnose is present in all control samples, but it is only present in the last time point sample of PS.

Pakulski (1986) previously measured sugar loss from *Spartina alterniflora*. Pakulski identified six monosaccharides lost in descending amount: mannose, ribose, galactose, fructose, rhamnose, and xylose. While he did not detect glucose or arabinose in the *Spartina* leachate, he detected ribose and fructose—two monosaccharides not detected in the present study. It is possible that these differences in findings are due to experimental design, for Pakulski's (1986) study was performed using live plants with attached leaves. In particular, Pakulski's (1986) failure to detect glucose is not surprising because photosynthate is easily shunted to rhizomes thus immobilizing it (Hopkinson and Schubauer 1984).

Glycosyl residues detected in the leachate are all carbohydrates present in *S. alterniflora* tissue (Opsahl and Benner 1999). In addition to the six glycosyl residues detected in the present experiments, Opsahl and Benner (1999) found lyxose, ribose, fucose, and myo-inositol in *Spartina* tissues. By comparing mole percents, glucose was the most abundant carbohydrate in *Spartina* tissues closely followed by xylose (Figure 4.2). Combined, they made up ~75% of total carbohydrate composition. In the leachate, however, we found that glucose and xylose made up < 43% of total carbohydrate composition. In contrast, arabinose, mannose, and galactose were found in lower concentrations in plant tissue than in the leachate.

V. Bacterial Cultures Receiving Leachate Additions

A. Bacterial Community

Bacterial counts presented in this thesis do not show much variation between treatments. Especially in Experiment I, bacteria numbers increased, but all populations crashed after 6 days of incubation. High cell density and the presence of protists were likely causes for this crash. A potential flaw in the experimental design was the preparation of the bacterial inoculum. We used size-fractionated environmental samples, which in itself is problematic. Filtration methods can reduce bacterial cell integrity, exclude particle-bound bacteria, select for smaller bacteria as the filter becomes clogged, and allow passage of non-target organisms such as viruses, protists, or fungi. The problems associated with filtration methods were recognized and addressed as best as possible by using low vacuum during filtration to reduce physical stress, frequently changing filter to reduce clogging, and incubating samples in the dark to reduce growth of photosynthetic, non-target organisms. However, a compounding factor was that the inoculum contained high concentrations of bacteria. Another way to design this experiment would have been to include a starting bacteria culture made from a dilution of the 1 μm natural water filtrate. Not only would this reduce initial bacteria numbers, it would also have reduced the numbers of non-target organisms. Other experimental biases would remain, but the likelihood of seeing a treatment effect or response from bacteria may have increased.

Bacterial OTU richness was significantly higher in cultures amended with leachate only (D). Previous studies have shown repeatedly that bacteria can use macrophyte DOC for growth (e.g. Findlay et al. 1986; Hullar et al. 1996). For example,

Hullar et al. (1996) found bacterial growth efficiency to be high (52%) when grown on *S. alterniflora* DOC. Similarly, Findlay et al. (1986) found that growth efficiency was much higher when bacteria were grown on macrophyte DOC (53%) than when grown on POC. This high community growth efficiency appears to be specific to DOC use. Therefore, it is possible that more bacteria are capable of using dissolved substrates in leachate.

P. spartinicola leachate appears to inhibit growth of several bacterial ribotypes (Figure 4.3). In Experiment II, γ -Proteobacteria and Gram-positive bacteria were identified in the cultures fed from Control but not in PS leachates. Those taxa were also absent in the PS and Natural treatments of Experiment I. However, when *P. spartinicola* and *Mycosphaerella* sp. 2 were in the same microcosm, bacterial populations growing in leachate include both γ -Proteobacteria and Gram-positive bacteria. It is possible that *P. spartinicola* is inhibitory to these ribotypes, and that this inhibition is reduced when *P. spartinicola* grows together with *Mycosphaerella* sp. 2. An alternative explanation is that fungi give some bacteria selective advantage over others.

The major bacterial OTUs identified by sequencing DGGE bands are similar to those previously found in the *S. alterniflora* decomposition system (Buchan et al. 2003). The majority of bands sequenced contained α -Proteobacteria (56%), followed by CFB (26%), γ -Proteobacteria (12%), then Gram-positive bacteria (6%; Table 4.3). Using cloning, culture, and TRFLP techniques, Buchan et al. (2003) found the bacterial decomposer community to consist of the same ribotypes in varying proportions. Even though the current study identified a lower percentage of α -Proteobacteria and higher percentages of CFB and γ -Proteobacteria associated with degrading *S. alterniflora*,

many of the DGGE bands in this study were not sequenced, thus it is possible that the non-sequenced ribotypes would make up the discrepancy in percentages.

B. Chitinase Activity

Chitinase activity displayed different trends depending on the experiment. Because the initial water samples were different (including bacterial populations, chemical composition, etc.), results of each experiment will be discussed separately. In general, however, chitinase activity was higher and less variable in Experiment II.

In Experiment I, microcosms with fungi alone had higher chitinase activity compared to the Empty and Natural treatments. Theoretically, these treatments would have produced more chitin, enhancing chitin degrading metabolism, so these results are not surprising. However, using that same logic, the D+C treatments that contained added chitin as a substrate should also display increased chitinase activity. This was not observed. In fact, chitinase activity decreased in D+C treatments.

There are several possible explanations for this discrepancy. The leachate contained large quantities of carbon and other substrates more labile than chitin, thus possibly decreasing the need for to use chitin as a carbon source. Chitinase expression might have been lower in cultures containing enough labile compounds to maintain bacterial growth, even if chitin was present. Alternatively, if bacteria were antagonistic to fungi, a possible mechanism of attack would be by expressing chitinase to break down fungal cell walls. In this scenario, chitinase activity would be high regardless of the availability of more labile organic matter in the leachate.

Therefore, results from Experiment I suggest that bacteria do not attack fungi using chitinase. Here, chitin added as a substrate did not induce the bacterial

community to express high chitinase activity. Another possible source of chitin to the bacterial cultures was the P+D treatment where pieces of fungal hyphae may be part of the particulate material in the rinse water. These treatments also displayed low chitinase activity throughout the experiment. However, it is possible that the MUF-substrate I used had a form of chitin not targeted by the chitinases in my cultures. If this is the true, than the MUF-incubations are invalid.

When comparing the effects of different Fungal treatments (PS, MS, PS+MS), *Mycosphaerella* sp. 2 appears to stimulate increased chitinase activity relative to *P. spartinicola*. Even though average chitinase activity was similar when bacteria were grown on P+D additions from PS, MS, or PS+MS, average chitinase activity was significantly higher in MS than PS when cultures were grown on D or D+C additions ($p\text{-value}_t < 0.01$). It is possible that some substance in the dissolved portion of *P. spartinicola* leachate (compared to *Mycosphaerella* leachate) inhibits bacterial chitinase activity.

In general, the fraction of leachate (P+D, D, or D+C) added as growth substrate did not significantly affect levels of chitinase activity. Only in the Natural treatment was chitinase activity significantly greater with P+D additions than with D additions alone ($p\text{-value}_t < 0.01$). Also, P+D additions generally caused chitinase activity to increase faster than other additions.

The opposite trend for expression of chitinase activity was found in Experiment II where higher activity was measured with P+D additions compared to D and D+C additions. The relatively low activity in the D treatment is consistent with the hypothesis

constructed from Experiment I: that the dissolved fraction of *P. spartinicola* leachate inhibited chitinase activity.

Contrary to our initial hypotheses, chitinase activity in enrichment cultures was generally higher when bacteria were grown in the absence of fungal leachate (Control). One possibility is that bacteria could degrade hyphal walls from non-living fungi more easily than hyphal walls from living fungi. Even though fungal biomass was significantly greater in the PS treatment, the method used only quantifies living fungal biomass since ergosterol degrades rapidly upon cell death. What we did not measure, then, was the non-living fungal content of the leaves. The leaves used in these experiments were taken from the natural environment, so they had been colonized by fungi prior to being harvested. So, even though autoclaved, the leaves likely contained hyphal walls from dead fungi. Therefore the increased chitinase activity in the Control could reflect the bacteria population's ability to degrade "inert" chitin.

VI. Conclusions

Plant vascular tissue decomposes slowly, on the order of years, so it is not surprising that the chemical properties of leaves measured in these experiments did not change dramatically over the course of the experiments. By the time standing dead leaves were collected, the *Spartina* plant had already shunted photosynthate to the rhizome, and the initial leaching phase had most likely occurred. The stage at which the leaves were collected and the slow rates of decomposition suggest that these experiments sampled the early stages of microbially-dominated decay.

Natural stands of *Spartina alterniflora* persist throughout the year, and it is common for dead leaves to remain attached to shoots from fall to spring. These dead

leaves support a continuous population of fungi capable of degrading lignocellulose. We had hypothesized that the presence of fungi would increase the production of particles because hyphae theoretically disrupt *Spartina* tissues. However, the presence of actively growing *P. spartinicola* significantly reduced the amount of particles that was produced from leaves, suggesting that hyphae hold leaves together. This may help explain the persistence of standing dead leaves in salt marsh areas. In fact, this may be an advantage for the fungi, because studies suggest that fungi are outcompeted for leaf material by bacteria as senescent leaves collapse to the sediment surface (e.g. Newell et al. 1989). Therefore, it would be advantageous for fungi to hold leaves together as long as possible so that they can degrade the leaves aerially and avoid strong competition with bacteria on the sediment surface.

The C:N of particles rinsed from leaves did not differ between treatments. This suggests that microbes do not alter the relative chemical composition of particles that initially enter detrital systems. In fact, data collected from the Georgia Coastal Ecosystem-Long Term Ecological Research group (GCE-LTER, Merryl Alber lab) from the Altamaha River estuary during 2001 shows that the average C:N of particulate matter (10.3 ± 1.6 ; collected on GF/F filters) is similar to the C:N ratio calculated from this study.

In my experiments, the amount of carbon, nitrogen, and phosphorus leached from *Spartina* leaves by rinse water decreased rapidly in treatments containing fungi. Because this rinse mimics natural wetting episodes such as tides or rain, my result indicates that mineralized or solubilized materials leached from decaying *Spartina* can be dispersed throughout the tidal marsh and carried out to the ocean through creeks.

It was not surprising that α -Proteobacteria were the dominant ribotypes observed in these experiments, considering that they are the dominant taxa in coastal Georgia waters (e.g. Gonzalez and Moran 1997) and in the *S. alterniflora* decomposition system (e.g. Buchan et al. 2003). There is also evidence that bacteria from this taxon may associate with the dominant ascomycete, *Phaeosphaeria spartinicola* (Buchan et al. 2003). If these associations are real, this may help explain the co-occurrence and dominance of *P. spartinicola* and α -Proteobacteria in the *S. alterniflora* decay system.

The results of these experiments provide evidence of possible fungal antagonism toward some species of bacteria. However, another interpretation is that fungi promote the development of a synergistic bacterial community that is best adapted to the presence and activity of fungi. In support of the latter interpretation, chitinase activity (presumed to be bacterial) decreased in cultures receiving *P. spartinicola* leachate additions (specifically the dissolved fraction) relative to other treatments. Therefore, some substance in the dissolved fraction of the rinse may have been responsible for suppressing the expression of chitinase in the cultures. If chitinase activity is taken as an indicator of bacterial antagonism toward fungi, suppression of bacterial chitinase activity could either indicate the effect of an active fungal antibiotic that inhibits chitinase expression or the development of a bacterial consortium with a mutualistic relationship to fungi, that do not express chitinase to prevent harming fungi.

Further evidence of an interaction between bacteria and fungi is that fewer bacterial OTUs were present when leachate from *P. spartinicola* microcosms was added to bacterial cultures. Again, it is possible that *P. spartinicola* produces antibiotics that inhibit the growth of specific bacterial OTUs. It is also possible that particular

bacterial taxa better adapted to co-existing with *P. spartinicola* have a selective advantage in my cultures, and thus dominate the ensuing assemblage. The second hypothesis is the more likely because an increasing number of studies are identifying associations between certain bacteria and fungi (e.g. Buchan et al. 2003). If fungi and bacteria are physically separated, as was the case in my microcosms, the main pathway by which fungi can influence bacteria is through dissolved substances. Further study is needed to assess if and how this would change if fungi and bacteria were growing in close proximity on standing dead *Spartina* tissue.

Table 4.1: Comparison of C:N ratios from leaves, particles, and leachate. Data were averaged over 32 days for Experiment I and 24 days for Experiment II. PS = *P. spartinicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartinicola* and *Mycosphaerella* sp. 2.

Exp	Treatment	C:N ^a		
		Leaves	Particles ^b	Leachate ^b
I	Natural	81 ± 5 ^c	9 ± 1	34 ± 12
	PS	85 ± 7	9 ± 4	36 ± 9
	MS	89 ± 4	10 ± 3	31 ± 9
	PS+MS	91 ± 5	10 ± 3	50 ± 24
II	PS	74 ± 4	11 ± 3 [*]	26 ± 9
	Control	78 ± 5	15 ± 4 [*]	43 ± 5

^a mole/mole

^b Atomic ratios were corrected for background carbon and nitrogen found in the negative controls (Empty treatment).

^c average ± standard deviation

^{*} No negative control was run for particle analysis during Experiment II, so particle information from the Empty treatment of Experiment I was used to correct for background carbon and nitrogen.

Table 4.2: Literature values of dissolved material leached from *Spartina alterniflora*. In the literature, dissolved organic carbon (DOC) lost as leachate was most often expressed as a rate ($\mu\text{g C (g dry weight)}^{-1} \cdot \text{h}^{-1}$). By making several assumptions, we estimated the leaching rates from the current study, and we erred on the side of underestimating DOC leaching rates. Previous experiments either incubated *S. alterniflora* leaves in salt marshes while leaves were still attached to the plant, or they used leaves cut from the plant. Experiments were conducted using various types and volumes of fluid and different incubation lengths.

Location	Plant material	Incubations	DOC ^a	C:N ^b	C:P ^b
Georgia, USA (Turner 1978)	attached, live	30 min. pre-soak	200 – 800 (n=33)		
Georgia, USA (Pakulski 1986)	attached leaves	200 mL, 2 hours	16 – 835 (n=18)		
Louisiana (Turner 1993)	attached, green leaves	tidal creek water, 3 hours; 30 min pre-soak	334 ± 249 (n=37)	16.1 ± 12.1 (n=13)	81 - 164
Atlantic coast, USA (Turner 1993)	attached, green leaves	tidal creek water, 3 hours; 30 min pre-soak	346 ± 213 (n=3)	8.3 ± 12.7 (n=3)	465 ± 320 (n=3)
Georgia, USA (Gallagher et al. 1976)	litterbags	150 mL tidal creek water, 3 hours;	42 – 163		
Georgia, USA (Hopkinson & Schubauer 1984)	cut leaves	250 mL tidal creek water, 2 hours	102.58 ± 12.0	10.2 ± 0.09	
Georgia, USA (current study) ^d	cut, standing dead leaves	90 mL tidal creek water rinse (~5 min)	419 ± 96 (n=18)	36.3 ± 9.2 ^c (n=18)	169 ± 113 ^c (n=18)
Georgia, USA (current study) ^d	Cut, autoclaved, standing dead leaves	90 mL tidal creek water rinse (~5 min)	4083 ± 953 (n=14)	42.7 ± 5.4 ^c (n=14)	158 ± 15 ^c (n=14)
Georgia, USA (current study) ^d	Cut, autoclaved, standing dead leaves inoculated with fungi	90 mL tidal creek water rinse (~5 min)	2183 ± 2038 (n=68)	38.9 ± 18.5 ^c (n=68)	208 ± 113 ^c (n=68)

^a leachate rates in $\mu\text{g C (g dry wt)}^{-1} \cdot \text{h}^{-1} \pm 1 \text{ SD}$

^b atomic ratios

^c assuming 22 g dry weight per microcosm and a 15 minute flushing time

^d corrected for background nutrient concentrations

Table 4.3: Percentage of major ribotypes identified in bacterial communities associated with *S. alterniflora* decay. Results from the current study are compared to those from Buchan et al. (2003) and show the presence of four groups of ribotypes: α - and γ -Proteobacteria; *Cytophaga*, *Flavobacterium*, and *Bacteroides* (CFB); and Gram-positive bacteria. Data represent the percentage of all identified OTUs corresponding to that ribotype.

Citation	Method	α -	CFB	γ -	Gram Positive
Buchan et al. (2003)	Clone library	81	4	4	0
Buchan et al. (2003)	Culture collection	82	2	2	13
Buchan et al. (2003)	T-RFLP	78	11	3	0
Current study	DGGE	56	26	12	6

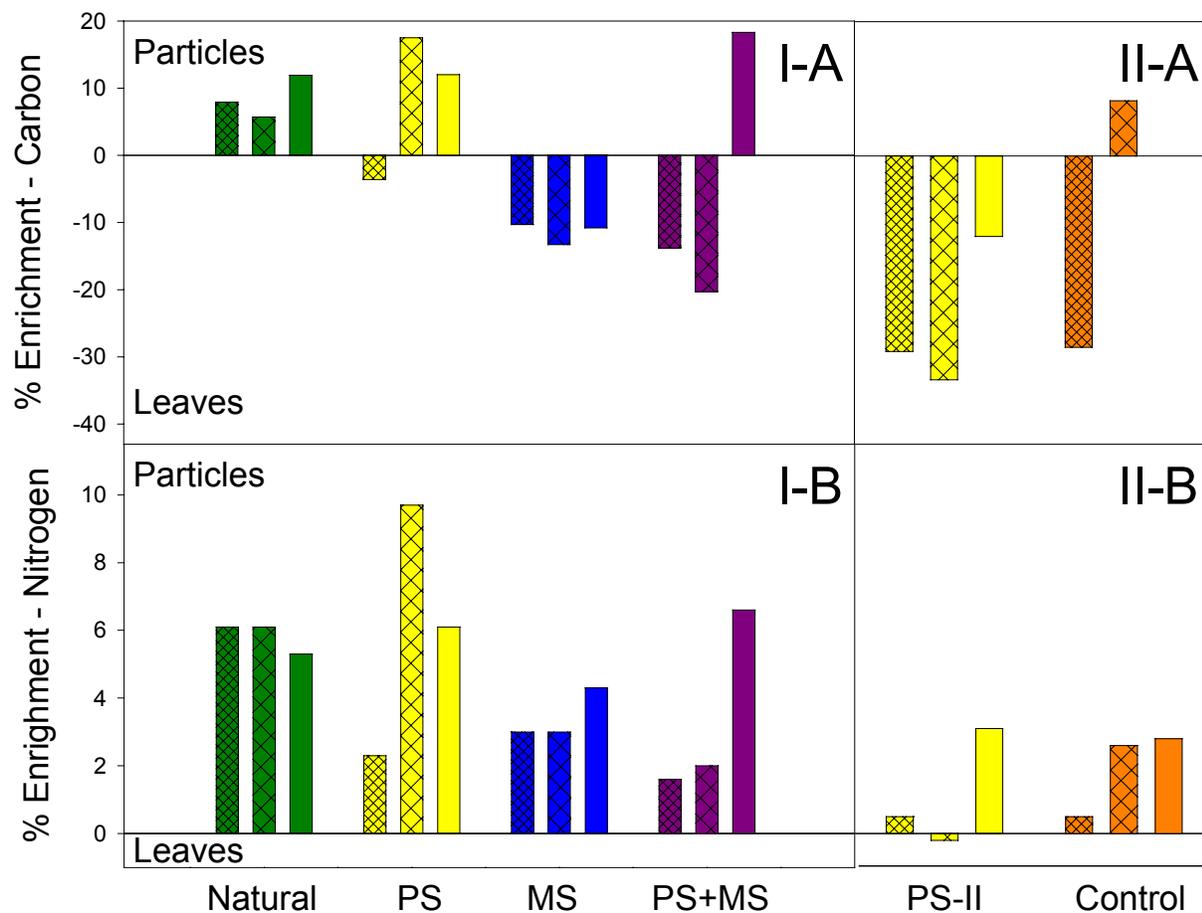


Figure 4.1: Difference in carbon and nitrogen content of leaves and the particles rinsed from leaves. Positive values indicate enrichment in the particles. Negative values indicate enrichment in the leaves. Tightly crossed bars are from initial samples (Day 0). Loosely crossed bars from samples collected at midpoint (Experiment I: 16 days after first rinse; Experiment II: 12 days after first rinse). Open bars are from samples collected at endpoint (Experiment I: 32 days after first rinse; Experiment II: 24 days after first rinse). (I) Experiment I. (II) Experiment II. (A) % Carbon enrichment of particles compared to leaves. (B) % Nitrogen enrichment of particles compared to leaves. PS = *P. spartnicola*, MS = *Mycosphaerella* sp. 2, PS+MS = *P. spartnicola* and *Mycosphaerella* sp. 2, and PS-II = *P. spartnicola* from Experiment II.

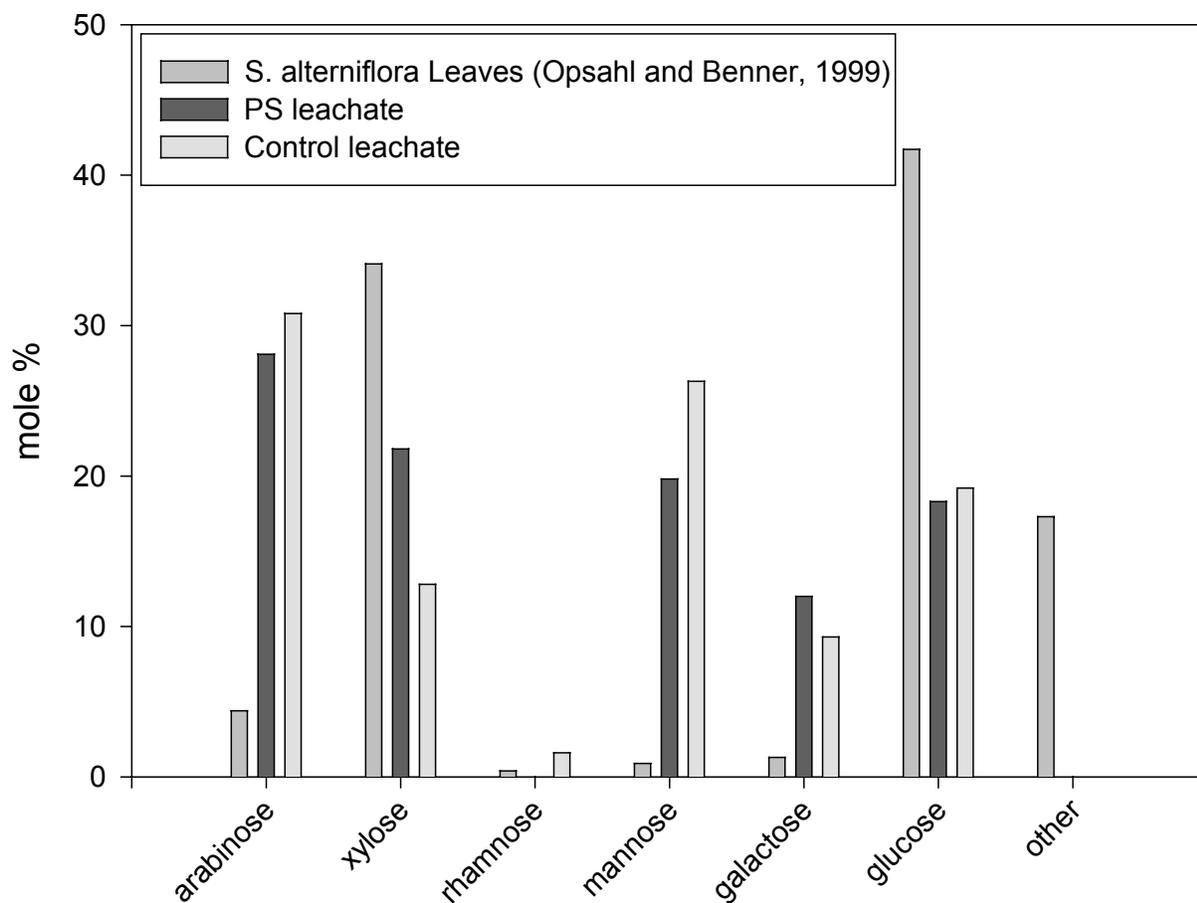


Figure 4.2: Comparison of carbohydrates found in *S. alterniflora* plant tissue and in leachate. Values for leaf tissue were calculated from Opsahl and Benner (1999) using the carbohydrate values reported for senescent leaves and converting into mole % composition using the molecular weight of each sugar. Leachate values were calculated from the same molecular weights and data from total carbohydrate analyses from my experiments. Leachate data were from samples collected on the initial (Day 0) sampling day. PS = *P. spartinicola*.

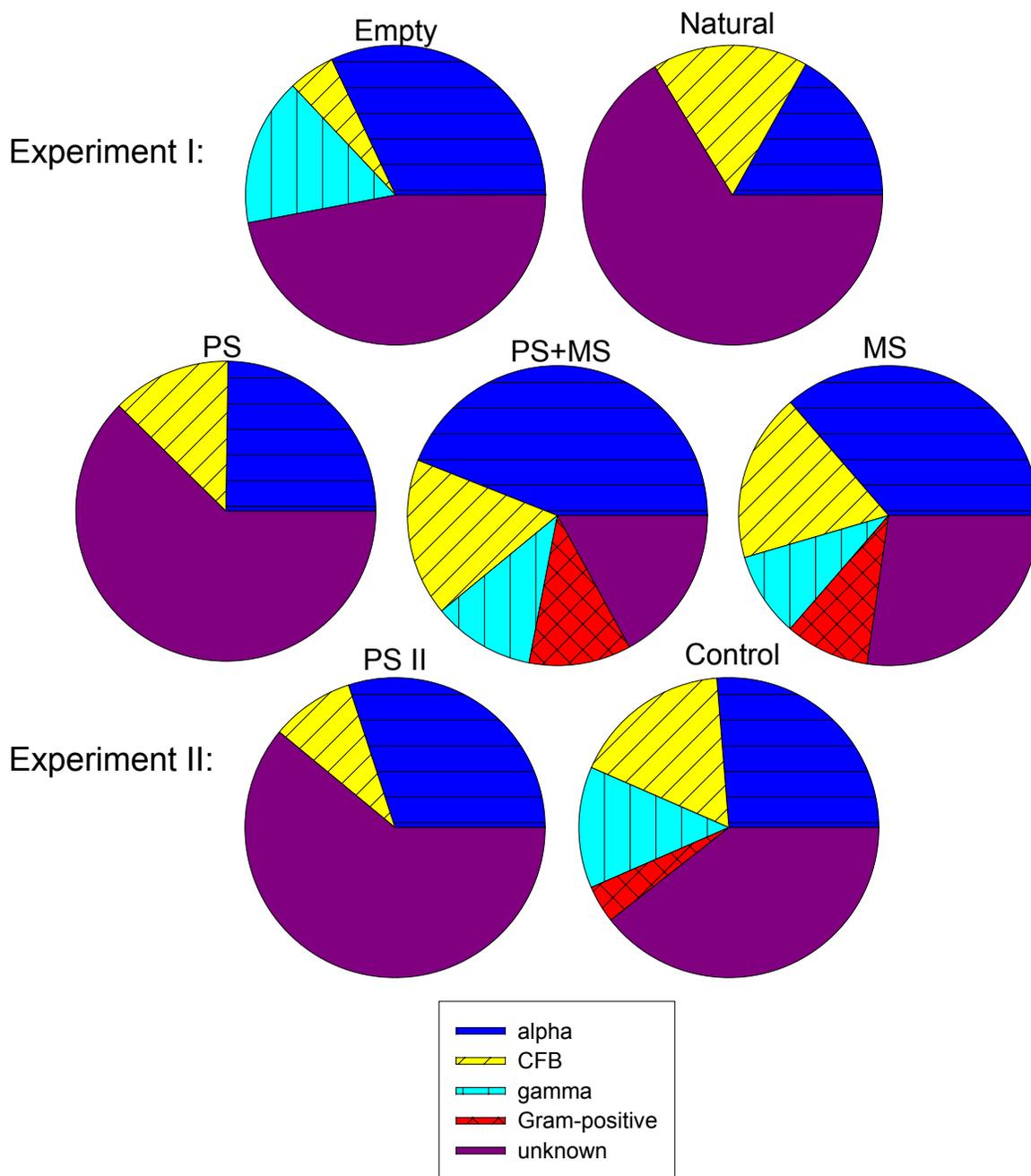


Figure 4.3: Relative ribotype composition of the bacteria in enrichment cultures. Coloration represents the proportion of OTUs affiliated with the given ribotype as determined by sequencing DNA in excised DGGE bands. Ribotypes identified include α -Proteobacteria; *Cytophaga*, *Flavobacterium*, and *Bacteroides* (CFB); γ -Proteobacteria; and Gram-positive bacteria. The identity of DNA fragments in the remaining bands was not determined.

CHAPTER 5
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