Molecular Phylogenetic Characterization of Microbial Community Dynamics Associated with Freshwater Stream Environmental Organic Matter Sources

by

Molli Michele Newman

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 12, 2011

Keywords: leaf breakdown; microbial community; succession; stream;
macroinvertebrates; anthropogenic disturbance

Approved by

Jack W. Feminella, Co-chair, Professor of Biological Sciences
Mark R. Liles, Co-chair, Associate Professor of Biological Sciences
Nedret Billor, Associate Professor of Mathematics and Statistics
B. Graeme Lockaby, Professor of Forestry and Wildlife Sciences

Abstract

The effects of various biotic and abiotic factors on microbial community dynamics during leaf breakdown were assessed through a series of *in situ* leaf breakdown studies within the Fort Benning Military Installation (FBMI), Georgia, USA. Leaf litter microbial community composition was mainly controlled by incubation time and, to a lesser extent, by leaf chemistry. Instream sediment disturbance and its associated effects on stream physicochemical conditions drastically altered bacterial assemblage composition during leaf breakdown.

Chapter 2 described a protocol for purifying genomic DNA from environmental sources for use in a polymerase chain reaction (PCR). This protocol was necessary because many of the techniques utilized in this dissertation involved extraction and amplification of genomic DNA from organic matter and often required purification of the genomic DNA. The protocol involves embedding genomic DNA extract in an agarose plug and incubation within a formamide and saline solution. The purified DNA can then be extracted from the agarose and used as a template for PCR. A test of this protocol using red maple leaf genomic DNA yielded significantly more amplicons using ~20 ng of purified DNA compared to extracted DNA alone.

Chapter 3 described a 128-d *in situ* leaf breakdown study within a single stream at FBMI to assess the effects of shredding macroinvertebrates on leaf

litter microbial communities. Contrasting mesh sizes (6.35- and 1-mm mesh) were used to reduce shredder macroinvertebrate abundance, and microbial community composition was characterized over 9 dates. Macroinvertebrate results revealed no reduction in shredder abundance, suggesting that the use of 1-mm mesh may be inappropriate in streams where the dominant shredders are fairly small and slender (e.g., *Polypedilum* and *Leuctra* spp.).

Chapter 4 described the differences in microbial community composition between leaf species of strongly contrasting leaf chemistries and associated breakdown rates. Maple and oak leaf species were used due to their drastically different leaf chemistries (e.g., higher percent lignin and cellulose in oak). Leaf chemistry differences resulted in significantly different microbial community composition measured using both ribosomal intergenic spacer analysis (RISA) and phospholipid fatty acid analysis (PLFA). These differences in microbial community composition were strongest during early leaf breakdown and decreased over time. Time was the main factor found to structure leaf litter bacterial assemblages with early and later breakdown times having different bacterial assemblage compositions.

Chapter 5 described a 64-d *in situ* leaf breakdown study at FBMI to quantify the effects of sediment disturbance on leaf litter bacterial assemblages. A variety of response variables were measured including several physicochemical conditions (streamwater temperature, pH, depth, current velocity), leaf breakdown, and bacterial assemblage composition (measured using RISA and bar-coded pyrosequencing). The main physicochemical

condition measured in this study that affected bacterial assemblage composition of leaf litter was streamwater pH, which was correlated to disturbance intensity, which in turn correlated to sediment. Overall, results showed sediment disturbance significantly altered leaf litter bacterial assemblage composition and was associated with a shift towards an assemblage capable of surviving harsher environmental conditions (e.g., increased pH, decreased dissolved oxygen).

Acknowledgements

I would like to thank my mentors, Drs. Jack Feminella and Mark Liles, for their continued guidance, support, and friendship during this dissertation work. I also thank my committee members, Drs. Nedret Billor and Graeme Lockaby, for always being supportive and providing assistance throughout this dissertation. I am also very grateful to Dr. Joseph Kloepper for serving as university reader. Many thanks to the following for contributing to both my development as a graduate student and the research within this dissertation: Nancy Capps, Dr. Abel Carrias, Erin Consuegra, Ann Marie Goode, Emily Hartfield, Laura Heck, Dr. Brian Helms, Miller Jarrell, Kavita Kakirde, Dr. Ely Kosnicki, Dr. Andrew Land, Brian Lowe, Jahangir Hossain, Dr. Kelly Maloney, Dr. Richard Mitchell, Shamima Nasrin, Dr. Larissa Parsley, Chao Ran, Susan Reithel, Brad Schneid, Stephen Sefick, and Malachi Williams. I would especially like to thank Brittni Bryant for her help and dedication in the laboratory. Funding from the U.S. Department of Defense's Strategic Environmental Research and Development Program (SERDP) and an Auburn University Graduate School research award supported this research. Thank you to my entire family for their support and encouragement during this endeavor. Most of all, I would like to thank my husband, Shelby Newman, for his continuous and loving support and understanding during this dissertation. I could not have done this without you.

Table of Contents

List of Tables

List of Figures

CHAPTER I

A. LITERATURE REVIEW

Allochthonous leaf litter inputs represent a significant energy source for stream ecosystems, especially within small, forested watersheds. In their classic study, Fisher and Likens (1973) demonstrated that ~99% of the energy fueling Bear Brook, at the Hubbard Brook Experimental Forest, New Hampshire, was from allochthonous sources. Of these inputs, leaf litter in particulate form composed 44.2% of the energy entering the stream, either through litter fall or wind transport. Leaf litter entering streams may act either as a nutrient source or sink, depending on ambient nutrient levels and the demands of stream organisms (Tate and Gurtz, 1986). These and other early studies (e.g. Minshall, 1967; Cummins, 1974) illustrated the importance of allochthonous leaf litter inputs to stream energy and nutrient flow, and sparked numerous subsequent studies investigating factors affecting the processes of leaf breakdown, and thus the release of energy and nutrients to the recipient ecosystem.

Leaf breakdown, or decomposition, is 'the combined result of physical and biological mineralization and transformation processes, resulting in the generation of CO_2 and other inorganic compounds, dissolved and fine-particulate organic mater (DOM and FPOM, respectively), and decomposer biomass' (Hieber and Gessner 2002). Historically, breakdown has been viewed as a

stepwise process consisting of 3 temporally distinct phases (Fig. 1.1), leaching,

microbial conditioning, and fragmentation (Petersen and Cummins, 1974;

Webster and Benfield, 1986; Boulton and Boon, 1991; Abelho, 2001). However,

in recent years, some have demonstrated that the phases of leaf breakdown

actually overlap and are not as distinctly separate as previously thought

(Gessner *et al.*, 1999).

Leaf litter (hereafter 'litter') typically enters the stream as coarse

particulate organic matter (CPOM) and begins *leaching*, where soluble organic

and inorganic components are released. During this portion of breakdown, litter

often is a nutrient source, releasing soluble sugars and polyphenolic compounds

(Nykvist, 1961; Suberkropp *et al.*, 1976). Typically, leaching occurs during the

first 24-48 h of incubation within a stream but can occur up to 7 d depending on

leaf species and other environmental variables (Nykvist, 1963; Canhoto and

Graca, 1996). Leaching can account for a rapid loss ranging from ~4-42% of

initial mass (Canhoto and Graca, 1996; Maloney and Lamberti, 1995). Leaching

effects on mass loss varies greatly among leaf species; thus, some researchers

have attempted to pre-leach litter to reduce variation in initial leaf breakdown

from leaching, although this practice often produces atypical breakdown rates

(Boulton and Boon, 1991). Oven drying of litter also can affect leaching through

its effects on leaf cuticular structure, which often increases breakdown rate

(Taylor and Barlocher, 1996).

Instream leaching of soluble litter components is followed by *microbial

colonization* and *conditioning*. At this point, litter is typically low in N but high in C,

which can serve as a substrate and food source for stream microorganisms (Cummins, 1974). Microbes consume this C-rich source, which, in turn, increases microbial biomass and litter quality for macroinvertebrates and other secondary consumers. As microbial biomass increases, nutrients are assimilated and transformed leading to increases in N content and nutrient quality of microbes (Kaushik and Hynes, 1971). As they grow and reproduce, microbes also produce several extracellular enzymes that can mediate microbial degradation of CPOM (Sinsabaugh *et al*., 1991), thus 'conditioning' litter. Sinsabaugh and Moorhead (1994) showed that enzymes involved in lignocellulose degradation and the cycling of nutrients, including N and P, were the most important to microbial conditioning of litter. The contribution of microorganisms to leaf mass loss is relatively low (~22-27%) compared to stream macroinvertebrates (~51-64%) (Hieber and Gessner, 2002). However, macroinvertebrate preference of litter is largely affected by microbial conditioning (Petersen and Cummins, 1974; Wright and Covich, 2005). Thus, although overall litter mass loss due directly to microbes is low, microbial conditioning is fundamentally important to litter colonization and high mass loss from macroinvertebrates and, thus, nutrient / energy release to higher trophic levels. Macroinvertebrates dependence on microbial conditioning as well as nutritional content in litter has long been known. Cummins (1974) analogized this dependence as the "peanut butter-cracker" relationship, highlighting the nutritional importance of microbes ("peanut butter") on nutritionally low-quality leaf litter ("cracker"). Presumably because of their ability to transform nutrients, microbes have been shown to play a role in

regulating macroinvertebrate feeding and nutrition (Cargill *et al*., 1985; Arsuffi and Suberkropp, 1989). Regardless of whether microbes influence breakdown by transforming nutrients, alter physical leaf structure, or simply consume litter for their own C source, it is clear that they play a major role in breakdown and degradation of leaf litter and the subsequent cycling of energy and nutrients.

Fragmentation is typically thought to occur after litter has been conditioned, which can be caused by shredding from stream macroinvertebrates (Wallace and Webster, 1996), as well as by physical abrasion from stress exerted by flowing water. Overall, fragmentation results in the physical conversion of residual CPOM to FPOM (Cummins, 1974; Allan, 1995). CPOM-consuming shredders often typically consist of macroinvertebrates in the aquatic insect orders Plecoptera, Trichoptera, and Diptera (Tachet *et al*., 1987; Wallace and Webster, 1996; Graca, 2001). Shredding macroinvertebrates can cause overall litter mass loss of up to 63% (Hieber and Gessner 2002) for some leaf species. Aside from the nutrient content obtained from degrading litter, shredders also may derive key limiting nutrients by consuming associated litter microbes, which have a higher nutritional content than unconditioned litter (Moriarty and Pullin, 1987), and can actually show preference for conditioned litter. Arsuffi and Suberkropp (1989) demonstrated macroinvertebrate preference for fungal-colonized litter by Diptera, Plecoptera, and Trichoptera, indicating that microbial assemblages can influence macroinvertebrate colonization and subsequent breakdown. As macroinvertebrates fragment leaf litter, it is further broken down into smaller particulate organic matter as well as digested and released back into

the stream environment as FPOM (Cummins, 1974). An additional source of FPOM comes from the flocculation of dissolved organic matter (DOM) following leaching and microbial assimilation (Cummins, 1974).

In terms of the factors affecting the timing relationship between litter leaching and microbial conditioning, there is likely some critical concentration of inhibitory compound(s) that, once leached, allows for colonization by previously inhibited microbes. Yet, other microbes may be inhibited by a different concentration of the compound and colonize at a later or earlier time. This differential response creates a process where microbial colonization and litter conditioning overlaps with leaching (Gessner *et al.*, 1999); in contrast, traditional studies may have viewed microbial colonization as a portion of a stepwise process that always followed leaching (e.g. Cummins, 1974). A similar situation likely is true for macroinvertebrate colonization and fragmentation where some, but not all, macroinvertebrates are capable of digesting certain leaf litter only after litter reaches some critical point of palatability. Then, based on the species-specific limitations of each macroinvertebrate group, successional colonization would occur with some, but not all, macroinvertebrates colonizing only after microbial conditioning. An example of this preferential colonization by stream macroinvertebrates following leaching was observed in a study by Pereira, *et al.* (1998) where they observed arthropod colonization following stabilization of polyphenol content. Leaching, microbial conditioning, and fragmentation all occur during leaf breakdown; however, these processes likely not only occur in an overlapping (vs. sequential) progression (Fig. 1.2a), but they also may account

for production of primary or secondary products that may regulate breakdown (Fig. 1.2b).

Given that microbes (including bacteria and fungi) contribute sizably to overall leaf mass loss during breakdown (Hieber and Gessner, 2002), studies have explored the separate contributions of microbial consumers to the process. Hieber and Gessner (2002) found that fungi contributed to ~15 and 18% of overall mass loss in alder and willow leaves, respectively (see also Gessner, 1997). In constrast, Hieber and Gessner (2002) found bacteria to contribute a leaf mass loss contribution of only ~13 (alder) and 9% (willow). These estimates were made after taking into account bacterial cell turnover rates as previous studies only measured bacterial biomass at a single point in time, and thus likely underestimated bacterial biomass lost from substrate (Hieber and Gessner, 2002). Regardless, most studies conclude that fungi exert a greater contribution to litter breakdown than bacteria (Suberkropp and Klug, 1976; Baldy *et al.*, 1995; Hieber and Gessner, 2002).

There is a wealth of evidence suggesting time-dependent colonization of instream litter by fungi and bacteria. Several studies have shown that fungi dominate microbial biomass during the initial phases of breakdown and decrease in biomass over time (Triska, 1970; Kaushik and Hynes, 1971; Suberkropp and Klug, 1976). Colonization of litter by fungi also has been shown to exhibit succession (below, see also Suberkropp and Klug, 1976). Although much lower than fungi, bacterial biomass tends to increase as fungal biomass decreases (Suberkropp and Klug, 1976; Weyers and Suberkropp 1996). This interaction

between fungi and bacteria has been described as an antagonistic relationship. By inoculating microcosms with either fungi or bacteria alone and a combination of bacteria and fungi, Mille-Lindblom and Tranvik (2003) observed higher biomass accumulation for both fungi and bacteria alone compared to in coexistence. Fungi appear more capable of colonizing freshly senesced litter because of their ability to form hyphae, which can penetrate the resistant leaf cuticle (Suberkropp and Klug, 1980) and provide a colonization benefit over bacteria. This advantage allows fungi to contribute more actively to breakdown during early phases; in contrast, bacteria are generally more active during later stages once fungi have degraded the leaf cuticle and other structural components.

Research on specific fungal taxa involved in litter breakdown has typically revealed dominance by aquatic hyphomycetes (Ascomycota) over terrestrially derived fungi. Early studies by Suberkropp and Klug (1976) found *Flagellospora curvula* Ingold and *Lemonniera aquatica* DeWild to be the dominant fungal species during the first 4-6 wk of breakdown of white oak and pignut hickory leaves. After 6-8 wk of incubation, there was a succession from early fungal species to *Alatospora acuminata* Ingold, *Tetracladium marchalianum* DeWild, and also *Anguillospora* sp. and *Clavariopsis aquatica* (Suberkropp and Klug 1976) in litter packs of white oak and pignut hickory. More recently, Nikolcheva and Barlocher (2004) found fungi of the division Ascomycota dominated linden, maple, beech, and birch litter. The fungal divisions Basidiomycota and

Chytridiomycota also were present, as well as Oomycota and Zygomycota in some seasons (Nikolcheva and Barlocher, 2004).

Comparatively less work has been conducted to study specific bacterial taxa present during litter breakdown than fungi, likely because bacteria contribute less to the breakdown process than fungi (Baldy *et al*., 1995; Hieber and Gessner, 2002). Early studies on microbial community structure found most bacterial cultured isolates were Gram-negative species from the genera *Flexibacter, Flavobacterium, Cytophaga, Achromobacter*, and *Pseudomonas* (Suberkropp and Klug, 1976). More recent work also has isolated bacteria from the *Cytophaga-Flavobacterium-Bacteroidetes* group as well as members of the phyla *Proteobacteria* and *Actinobacteria* (Das *et al*., 2006). Suberkropp and Klug (1976) was unable to culture members of the *Actinobacteria* phylum in their study, but this discrepancy could be because of methodological limitations. For example, the above study used dilutions plated onto PYG agar with an incubation of 2 wk for growth of viable bacterial cells, which was a very common medium and at the time was known to yield the highest viable cell counts and diversity. However, many bacterial taxa require >2 wk for cultivation (Janssen *et al*., 2002) and often have complex metabolic requirements unknown at the time of the previous study (see below, Amann *et al*., 1995). In addition, methodological limitations, including those mentioned above, may have prevented identification of other litter microbial community members.

Quantitative methods used to examine stream microbial community composition within litter have varied greatly and, in so doing, provided

researchers varying degrees of resolution on questions involving community structure. Early on, studies focused on quantifying overall microbial respiration and enzymatic activity and estimating microbial densities by culturing and direct cell counts (Witkamp, 1963; Suberkropp and Klug, 1976; Wallace *et al.*, 1982). These traditional approaches allowed direct quantification of microbes and made possible examination of leaf species differences in microbial density at single- and multiple-points in time during breakdown. However, direct cell counts provide no information about cell viability, whereas use of microscopical and staining techniques allowed a better visualization of microorganisms within the litter substrate. For example, use of 4',6-diamidino-2-phenylindole (DAPI) and 5-cyano-2,3-ditolyltetrazolium chloride (CTC) stains coupled with fluorescence microscopy allows viable and nonviable cells to be distinguished, and thus provides a more accurate estimate of cell densities and biomass in litter (Simon, 2000).

Historically, determining identity of fungal and bacterial taxa has relied on culturing techniques to grow the microorganisms present coupled with tests to characterize and identify them (Suberkropp and Klug, 1976). Within the past 10 y researchers have recognized that ~99% of microorganisms have complex growth requirements and unpredictable nutritional demands that render them incapable of culture (Amann *et al*., 1995). This limitation has led to developing culture-independent techniques for analyzing microbial community structure and composition. These culture-independent methods allow for examination of microbial community structure and composition at various levels of resolution,

including from DNA sequencing to whole community similarity/dissimilarity (Findlay, 2010).

In a recent review of stream microbial ecology, Findlay (2010) described the varying levels of resolution provided by a full suite of methods for quantifying microbial community structure and composition. For broad levels, such as shifts in microbial community structure, DNA fingerprinting techniques including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (RISA), and terminal restriction-fragment length polymorphism (tRFLP) provide genetic profile-based information about community similarity patterns. Most of these methods provide minimal to no sequence information regarding the taxa present. The major benefit of these fingerprinting techniques is to characterize broad scale shifts in community structure without specific prior knowledge of taxa. Many studies have used these modern techniques alongside traditional methods for assessing microbial diversity and activity. This combined approach has helped address questions about seasonal, leaf species, and environmental effects on microbial communities in many aquatic habitats (Das *et al.*, 2006; Hullar *et al.* 2006; Rees *et al.*, 2006).

Relative to the above methods, sequencing of small ribosomal subunits (such as the 16S rDNA gene in bacteria or the 18S rDNA gene in fungi) has provided even finer resolution of taxa composition in microbial communities (Hullar *et al.*, 2006). Using methods including cloning as well as next-generation sequencing, researchers can now sequence and identify all members within a

microbial community, from species- to division-level, with the degree of confidence in taxonomic assignment increasing with sequence length (Huse *et al.*, 2007; Wommack, Bhavsar and Ravel, 2008). In this context, Findlay (2010) pointed out that division- or taxa-specific probes can be designed to screen for microbes within samples and also estimate their relative proportions. By combining traditional and contemporary (molecular-based) techniques, there is high potential to address questions regarding microbial community succession and response to contrasting environmental conditions that heretofore have not been possible.

Many of these above techniques are based on polymerase chain reaction (PCR) amplification of the environmental sample. Here, deoxyribonucleic acid (DNA) is directly extracted, with a specific portion of it being amplified with a specific primer pair. Depending on the sample, there may be substances co-extracted along with DNA that can inhibit further enzymatic-dependent downstream processes. The polymerase enzyme involved in PCR often can be inhibited from the presence of polyphenols, humic acids, and polysaccharides; each of these substances can occur in degrading leaf litter. In addition, many studies involving genomic DNA extraction from environmental samples commonly use DNA extraction kits (Roose-Amsaleg *et al.*, 2001). Several of these often involve alcoholic precipitation of DNA (Porteous *et al.*, 1997) and can co-precipitate humic substances, which have been shown to be highly inhibitory towards polymerase enzymes (Tebbe and Vahjen, 1993).

Genomic DNA extracted from litter usually requires further purification prior to PCR because of humic and phenolic substances and other low-molecular-weight contaminants (Widmer *et al.*, 1996; Widmer *et al.*, 1997). Presence of these co-extracted contaminants in extracted genomic DNA requires sample DNA purification following extraction. Several purification methods exist in the literature (reviewed by Roose-Amsaleg *et al*., 2001). Some of these methods involve addition of products, such as polyvinylpolypyrrolidone (PVPP), sodium ascorbate and hexadecyltrimethylammonium bromide (CTAB), during cell lysis that bind to humic substances, thus reducing their impact on downstream processes (Roose-Amsaleg *et al*., 2001). Often, following these treatments, it is still necessary to dilute genomic DNA and its co-extracted contaminants to an optimum that reduces contaminant concentration to a non-inhibitory level. However, in many instances these products and subsequent dilution are not sufficient and further purification is necessary. Some commonly used purification strategies include cesium chloride (CsCl) density gradient centrifugation, chromatography, electrophoresis, and dialysis and filtration. Samples high in humic substances, such as soils and organic matter-rich samples, often are electrophoresed in low-melt agarose so that faster migrating humic substances will separate from genomic DNA (for review, see Roose-Amsaleg *et al.*, 2001). This separation of contaminant and genomic DNA allows for the genomic DNA to be extracted from the agarose and used in subsequent analyses.

Many of the above methods result in a decrease in DNA yield, which can reduce efficacy of downstream processes, including products of PCR (Lear *et al.*,

2010). In addition, a single purification does not completely remove all contaminants, which then may require use of two (or more) purification methods. Using a combination of purification methods even further increases potential loss of DNA and its concentration. Selection of a purification method is often based on balancing the conflicting demands of increased processing time and reduced DNA yield (Roose-Amsaleg *et al.*, 2001). Thus, developing new PCR-based techniques capable of higher resolution detection is necessary to further purify extracted genomic DNA without the concomitant loss of DNA product.

In addition to the role of microbial communities in litter breakdown in streams, several other factors, including abiotic and biotic factors, can influence breakdown rate. Concentrations of dissolved nutrients, such as N and P, can alter leaf breakdown rates. Laboratory stream microcosm studies have shown NO_3-N and PO_4 additions stimulate microbial activity, and increase leaf fragmentation and breakdown rate (Howarth and Fisher, 1976). In situ studies have also shown strong effects of nutrients on litter decomposition. Streamwater P and N have been positively correlated with increased decomposer activity and leaf breakdown (Elwood *et al*. 1981; Suberkropp and Chauvet, 1995; Gulis and Suberkropp, 2003). Increased concentrations of dissolved N and P potentially stimulate microbial activity and increase biomass, which, in turn, increases breakdown (Suberkropp and Chauvet, 1995; Gulis and Suberkropp, 2003). Breakdown also can be influenced by streamwater pH, often being higher in circumneutral (vs. acidic) stream water, where it is thought that low pH inhibits microbial growth (Webster and Benfield, 1986; Riipinen, *et al*., 2009).

High streamwater temperature has historically been associated with increased breakdown (Webster and Benfield, 1986), although this effect may be because of higher stream metabolism (e.g. gross primary production [GPP] and ecosystem respiration) (Irons, *et al*., 1994; Young *et al*., 2008). Increased temperature typically leads to increased GPP, which provides energy for primary consumers (Larsson and Hagstrom, 1979) and increased microbial respiration. Dissolved oxygen concentration within stream water may exacerbate the effects of temperature on metabolism, which if low, may decrease breakdown. Marmonier *et al.* (2010) found lower breakdown rates in sites with decreased dissolved oxygen; they hypothesized that lower breakdown rates in their sites could be explained by decreased oxygen availability that supports heterotrophic activity and alters invertebrate assemblage composition.

Aside from the above physicochemical conditions, inherent chemical and structural properties of the leaf litter itself also can affect breakdown. Melillo *et al.* (1983) found leaf breakdown to be slower in litter with lower N content, which was inversely related to the initial lignin content. In general, high concentrations of refractory structural components such as lignin have been considered the primary factors determining breakdown (Gessner and Chauvet, 1994; reviewed by Gessner, 2005). The effect of high lignin:N ratios, as well as high C:N and C:P, in decreasing leaf breakdown was also reported (Enriquez *et al.*, 1993).

Structural components of litter, such as lignin, cellulose, and hemicellulose, often are indigestible for stream macroinvertebrates who lack the appropriate digestive enzymes (Wright and Covich, 2005). For this reason, litter

high in structural components, particularly lignin, is generally unpalatable and of low quality to macroinvertebrates (Cummins and Klug, 1979). For leaf litter high in lignin, a large portion of the initial breakdown is from stream microorganisms that produce enzymes, such as cellulases, capable of lignin degradation (Pusch *et al*., 1998). Microbial conditioning increases litter palatability, and accumulation of microbial biomass increases nutritional quality of litter allowing further fragmentation from macroinvertebrates (Wright and Covich, 2005; reviewed by Graca and Zimmer, 2005).

Secondary plant compounds, including polyphenolics such as tannins, also may decrease breakdown. Studies have examined if this decrease occurs by colonization inhibition of stream macroinvertebrates and/or microbes. Canhoto and Graca (1995 and 1999) found that leaf consumption by the cranefly *Tipula lateralis* was negatively correlated with polyphenolic content. They also noted that those *T. lateralis* consuming leaves high in polyphenolic content, including eucalyptus and oak, did not grow. Macroinvertebrates may avoid consuming litter high in polyphenolics as part of their life history strategy to enhance growth. Canhoto and Graca (1999) showed that the inhibitory effects of secondary compounds on macroinvertebrate feeding could be transferred from eucalyptus leaves to fast-degrading leaf species. They also showed that aquatic hyphomycete growth decreased with increasing concentrations of the secondary compounds eucalyptus oil and tannic acid (Canhoto and Graca 1999). Previous studies also have suggested that secondary compounds inhibit microbial colonization of litter (Stout 1989), although this effect does not seem to occur in

the tropics. In a Costa Rican stream Ardon and Pringle (2008) found that secondary compounds were rapidly leached from 8 leaf species and were believed to be less important than structural compounds in determining breakdown.

In general, few studies have examined the effects of leaf species (and their associated structural and secondary components) on microbial community composition. Recently, Das *et al*. (2007) compared overall diversity of fungi, bacteria, and actinomycetes on 2 leaf species (sugar maple and white oak), and found time of exposure to be the major factor controlling microbial community composition. However, they used only DGGE banding patterns to characterize the communities, which unfortunately allows for co-migration of DNA fragments from different taxa to the same position on a gel, and they obtained no sequence information on dominant taxa. More definitive sequence-based research needs to be done to examine differences in microbial community composition in response to leaf chemistry differences at a finer level of taxonomic resolution. Having finer-scale resolution of the specific taxa present throughout the breakdown process would allow researchers to explore whether specific microbial taxa often were associated with faster- or slower-degrading species. In addition, it would allow for a direct comparison with previous studies describing the dominant taxa associated with degrading litter.

As mentioned previously, feeding and fragmentation of instream litter by shredders and other macroinvertebrate consumers can affect leaf breakdown (Barnes *et al*., 1986; McArthur and Barnes, 1988; Wallace and Webster, 1996).

Whole-stream insecticide treatment at the Coweeta Hydrologic Laboratory, North Carolina, USA provided strong evidence of the role of macroinvertebrates (Cuffney *et al.* 1990). Following a drastic reduction (>1,000,000 organisms/wk) of macroinvertebrates, up to a 74% reduction in breakdown occurred in the treated sections with no effect on bacterial density or microbial respiration. Whole-stream experiments such as this are usually not feasible, so most of the evidence directly linking macroinvertebrates and breakdown has been done on smaller spatial scales.

More commonly, researchers have manipulated macroinvertebrate abundance by excluding them from litter in mesh bags of different mesh sizes. Generally, a coarse mesh (~ 5.0mm) is used as a control to allow for macroinvertebrate colonization, whereas a finer mesh (~1.0mm) serves as the exclusion treatment (Boulton and Boon, 1991). For example, Stewart (1992) used mesh exclusions to assess the effect of macroinvertebrates on decomposition rates for several leaf species bordering woodland streams of southern Africa. In that study, macroinvertebrate effects on breakdown varied with shredder density, which varied among sites. At the site with the highest shredder density, Stewart (1992) observed breakdown rates of all leaf species examined were significantly faster in coarse (control) than fine (exclusion) mesh. Macroinvertebrate effects on breakdown were not present at sites with lower shredder densities (Stewart, 1992). Benfield and Webster (1985) found a similar result in an Appalachian stream where species-specific leaf breakdown rates varied with shredder abundance. More recently, the presence of shredders has

been implicated as a critical factor influencing leaf processing in streams (Sponseller and Benfield, 2001). Studies have examined the contribution and community composition of stream microbes and macroinvertebrates associated with leaf litter, although to date no studies have been designed to quantify the effects of macroinvertebrates on the microbial communities associated with litter in streams.

Disturbance plays a major role in stream community organization, whose effects can be both direct and indirect (Resh *et al*., 1988; Maloney and Weller, 2011). Disturbance is generally defined as 'any relatively discrete event in time that is characterized by a frequency, intensity, and severity outside of predictable range, and that disrupts ecosystem, community, or population structure and changes resources or the physical environment' (Resh *et al*., 1988). In stream ecosystems, disturbance can be natural or anthropogenic. Major examples of natural disturbance in streams are typically related to hydrologic regime, such as floods and droughts (Lake, 2000). Alternatively, anthropogenic disturbance is defined as 'any human-mediated event or activity that is virtually unknown in natural systems in terms of type, frequency, intensity, duration, spatial extent, or predictability over the last century' (Naiman *et al.*, 2005). Anthropogenic disturbance can result from many land use activities including, but not limited to, acid mining, agricultural practices, timber harvesting, and urbanization (Allan, 2004). According to the US Census Bureau, the US population is expected to increase by ~ 21% over the next 2 decades (U.S. Census, 2008). Increased land use is often associated with increased human population growth (Allan, Erickson

and Fay, 1997). Thus, increases in human population size of this magnitude are likely to increase occurrence of human-mediated disturbance in many stream ecosystems.

Human-mediated changes to the landscape within watersheds can alter instream hydrology and geomorphology, causing altered flow regime, channel shape, and increased sediment erosion and deposition within the channel (reviewed by Allan, 2004). Increased sedimentation can alter or degrade many instream variables, including stream habitat and the associated benthic food web (Henley *et al*., 2000; Allan, 2004; Downes *et al*., 2006;). By altering the stream bed, sedimentation may lead to decreased benthic habitat heterogeneity by infilling of interstitial spaces (Lake, 2000; Allan, 2004). Habitat heterogeneity (complexity) has been investigated in several studies for its role in structuring the benthic community. Habitat alteration can limit those organisms capable of colonizing a given location (Poff, 1997). Sedimentation has been shown to significantly influence species richness and diversity in aquatic insect assemblages (Lemly, 1982), and the overall structure of a benthic habitat can significantly affect species diversity and abundance (Downes *et al*., 1998). As such, disturbance can lead to a homogenized stream reach colonized primarily by relatively tolerant biota that are more suited to altered habitat than less tolerant species (Helms *et al.*, 2009). Overall, sediment disturbance has a high potential to yield an altered, more homogenous, and less diverse biotic community (Harrison, 2007).

Sediment disturbance can affect aquatic invertebrate population size and community structure in multiple ways. Aquatic invertebrate density and diversity are often directly related to substrate diversity (Gore, 1985). The effects of sediment on habitat heterogeneity can reduce habitat availability for some invertebrates and also increase their susceptibilty to predation (Newcombe and Macdonald, 1991). Beyond its influence on habitat availability, sediment also can affect invertebrate functional feeding group composition and abundance. For example, because of its effects on primary producer biomass and composition, sediment can alter abundance of secondary consumers, such as invertebrate grazers (Newcombe and Macdonald, 1991). Sedimentation can also affect invertebrate collector-filters, by clogging feeding structures, or even gills of non-filter feeders, leading to reduction in feeding efficiency and growth and increased mortality (Hynes, 1970; Lemly, 1982). In sandy coastal plains streams anthropogenic disturbance also can lead to impaired stream metabolism through reduced respiration and primary production (Houser *et al.*, 2005). These reductions separately or in combination can affect leaf breakdown rates by altering the rates at which leaf conditioning, fragmentation, and/or macroinvertebrate consumption occur.

Instream environmental measures, such as those used to assess water quality, are greatly affected by sedimentation. Streamwater temperature, turbidity, and dissolved oxygen available in stream water are among the main notable factors affected (reviewed by Ryan, 1991). Increased turbidity, along with an overall increase in suspended sediment load, is a common result of increased

sedimentation (Lemly, 1982). Elevated water temperature often is associated with high sedimentation, where radiation-stored sediment erodes into a stream channel increasing the water temperature (Hagans *et al*., 1986). As water temperature increases, there is also an associated decrease in the concentration of dissolved oxygen. Much research has been done to elucidate the response of macroinvertebrates to instream sedimentation, but comparatively little research has been conducted on the effects of sedimentation on the microbial community associated with leaf litter. Given that microbial conditioning must occur before macroinvertebrates generally colonize and consume litter, it is important to examine the degree to which sedimentation affects microbial communities associated with instream litter.

The effects of sedimentation on leaf breakdown have been equivocal. Benfield *et al*. (2001) found increased sedimentation immediately following the start of logging decreased leaf breakdown. Other studies concluded that decreased breakdown from sedimentation occurred because of burial of litter, which created an anoxic environment preventing fungal and macroinvertebrate colonization (Webster and Waide, 1982). In other situations, increased breakdown rates after logging, may cause increased stream flow and erosion of finer sediment, leaving behind coarser sediment that increased litter abrasion and fragmentation (Benfield *et al*. 2001). In a related study, Sponseller and Benfield (2001) found leaf breakdown rate was positively correlated with substrate particle size. In this context, depending on substrate particle size, sediment can either bury litter and reduce breakdown, or increase abrasion and

accelerate breakdown. Aside from burial impacts, sedimentation and its subsequent effects on instream habitat, as mentioned above, can reduce secondary consumer composition, which also can in turn reduce breakdown rates (Sponseller and Benfield, 2001). These interactive effects between physical factors (e.g. sedimentation) and stream organisms can lead to drastic alterations in leaf breakdown and energy release into stream food webs. However, little is known regarding their effects on leaf litter-associated microbial community composition. Knowing how sediment disturbance affects microbial community composition of litter could provide further insight into the mechanism by which sedimentation alters leaf breakdown.

B. SUMMARY

Allochthonous leaf litter provides a fundamentally important source of nutrients and energy inputs for stream ecosystems. Therefore, litter breakdown and concomitant release of these inputs from terrestrial sources is vital to fueling stream ecosystems. Typically, leaf breakdown studies involve incubation of leaf litter in situ and monitoring changes over time both in amount and quality of remaining leaf litter. Leaf breakdown, whether viewed as a stepwise process or overlapping phases, involves 3 processes: 1) leaching, 2) microbial conditioning, and 3) fragmentation. Of these, microbial conditioning is particularly important because of the dual role of microbes in nutrient transformation and in increasing litter quality and preference for macroinvertebrates. My dissertation focuses on investigating several aspects of leaf breakdown, in particular processes affecting microbial conditioning processes.

Because of data indicating a larger contribution by fungi than bacteria during breakdown, several studies have examined the specific fungal taxa involved in leaf breakdown. Less work has been done studying bacterial taxa present during breakdown. Recent advances in methodology allow for the examination of microbial communities at varying levels of resolution, ranging from very broad levels (e.g., fingerprinting techniques) to finer levels of resolution (e.g., next-generation sequencing). My dissertation decribes a pluralistic approach using traditional and modern molecular methods to characterize the stream microbial community associated with leaf litter breakdown, particularly in reference to how communities vary temporally and in response to differences in macroinvertebrate shredder abundances and leaf chemistry. Many of these techniques employ the use of Polymerase Chain Reaction (PCR) making them susceptible to inhibition from co-extracted enzymatic inhibitors. Several purification methods exist, but vary in efficiency based on sample source, cost, processing time, and purified genomic DNA yield. I developed and tested a low-cost, rapid method to further purify extracted genomic DNA that causes minimal concomitant loss of genomic DNA concentration.

Although biotic factors such as leaf litter chemistry and macroinvertebrates have been shown to affect leaf breakdown, few studies have examined the degree to which these consumers affect the associated microbial community. Both natural and anthropogenic (human-mediated) disturbance can drastically affect stream ecosystems, and with projected increases in the human population, anthropogenic disturbance is likely to greatly increase in the near future. One

form of anthropogenic disturbance of particular importance in stream ecosystems is land-derived sedimentation, which can affect both instream habitat and associated stream organisms. Sedimentation can both increase and decrease rate of breakdown, likely because of the varied effects of substrate particle size, and can also alter secondary consumer composition. Yet, surprisingly little is known about how sedimentation affects leaf litter-associated microbial community composition.

My dissertation research is separated into 4 main chapters, with the first data chapter (Chapter II) describing a method for purifying genomic DNA extracted from environmental sources for later use in PCR. Chapter III focuses on the effects of stream macroinvertebrates on leaf litter microbial communities *in situ* by reducing macroinvertebrate shredder abundance within leaf litter during breakdown. Chapter IV examines the effects of different leaf species on litter breakdown and how this effect alters microbial succession on litter. The last chapter (Chapter V) describes an additional leaf breakdown study designed to examine the effect of sediment disturbance on leaf litter microbial communities.

C. LITERATURE CITED

Abelho, M. 2001. From litterfall to breakdown in streams: a review. *The Scientific World Journal*, 1, 656-680.

Allan, J.D. 1995. *Stream ecology: structure and function of running waters*. Chapman and Hall. London, UK.

Allan, J.D. 2004. Landscapes and riverscapes: the influence of land use on stream ecosystems. *Annual Review of Ecology, Evolution, and Systematics*, 35, 257-284.

Allan, D., Erickson, D. and Fay, J. 1997. The influence of catchment land use on stream integrity across multiple spatial scales. *Freshwater Biology,* 37, 149-161.

Amann, R.I., Ludwig, W. and Schleifer, K.H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology and Molecular Biology Reviews*, 59, 143-169.

Ardon, M. and Pringle, C.M. 2008. Do secondary compounds inhibit microbial- and insect-mediated leaf breakdown in a tropical rainforest stream, Costa Rica? *Oecologia*, 155, 311-323.

Arsuffi, T.L. and Suberkropp, K. 1989. Selective feeding by shredders on leaf-colonizing stream fungi: comparison of macroinvertebrate taxa. *Oecologia*, 79, 30-37.

Baldy, V., Gessner, M.O. and Chauvet, E. 1995. Bacteria, fungi and the breakdown of leaf litter in a large river. *Oikos*, 74, 93-102.

Barnes, J.R., McArthur, J.V. and Cushing, C.E. 1986. Effect of excluding

    shredders on leaf litter decomposition in two streams. *Western North*

    American Naturalist, 46, 204-207.

Benfield, E.F. and Webster, J.R. 1985. Shredder abundance and leaf breakdown

    in an Appalachian Mountain stream. *Freshwater Biology*, 15, 113-120.

Boulton, A.J. and Boon, P.I. 1991. A review of methodology used to measure leaf

    litter decomposition in lotic environments: time to turn over an old leaf?

    Marine and Freshwater Research, 42, 1-43.

Bournaud, M., Tachet, H. and Roux, A.L. 1987. The effects of seasonal and

    hydrological influences on the macroinvertebrates of the Rhone River,

    France. II. Ecological aspects. *Archiv für Hydrobiologie*, Supplement, 76,

    25-51.

Canhoto, C. and Graca, M.A.S. 1995. Food value of introduced eucalypt leaves

    for a Mediterranean stream detritivore: *Tipula lateralis*. *Freshwater*

    Biology, 34, 209-214.

Canhoto, C. and Graáa, M.A.S. 1996. Decomposition of *Eucalyptus globulus*

    leaves and three native leaf species *Alnus glutinosa*, *Castanea sativa*

    and *Quercus faginea* in a Portuguese low order stream. *Hydrobiologia*,

    333, 79-85.

Canhoto, C. and Graáa, M.A.S. 1999. Leaf barriers to fungal colonization and

    shredders *Tipula lateralis*. consumption of decomposing *Eucalyptus*

    globulus. *Microbial Ecology*, 37, 163-172.

Cargill, A.S., Cummins, K.W., Hanson, B.J. and Lowry, R.R. 1985. The role of lipids, fungi, and temperature in the nutrition of a shredder caddisfly, *Clistoronia magnifica*. *Freshwater Invertebrate Biology*, 4, 64-78.

Coleman, M.J. and Hynes, H.B.N. 1970. The vertical distribution of the invertebrate fauna in the bed of a stream. *Limnology and Oceanography*, 15, 31-40.

Cuffney, T.F., Wallace, J.B. and Lugthart, G. 1990. Experimental evidence quantifying the role of benthic invertebrates in organic matter dynamics of headwater streams. *Freshwater Biology*, 23, 281-299.

Cummins, K.W. 1973. Trophic relations of aquatic insects. *Annual Review of Entomology*, 18, 183-206.

Cummins, K.W. 1974. Structure and function of stream ecosystems. *BioScience*, 24, 631-641.

Cummins, K.W. and Klug, M.J. 1979. Feeding ecology of stream invertebrates. *Annual Review of Ecology and Systematics*, 10, 147-172.

Cummins, K.W., Spengler, G.L., Ward, G.M., Speaker, R.M., Ovink, R.W., Mahan, D.C. and Mattingly, R.L. 1980. Processing of confined and naturally entrained leaf litter in a woodland stream ecosystem. *Limnology and Oceanography*, 25, 952-957.

Das, M., Royer, T.V. and Leff, L.G. 2006. Fungal, bacterial and actinomycete diversity on leaves decomposing in a stream. *Applied and Environmental Microbiology*, 73, 756-767.

Downes, B.J., Lake, P.S., Glaister, A. and Bond, N.R. 2006. Effects of sand

    sedimentation on the macroinvertebrate fauna of lowland streams: are

    the effects consistent? *Freshwater Biology*, 51, 144-160.

Downes, B.J., Lake, P.S., Schreiber, E.S.G. and Glaister, A. 1998. Habitat

    structure and regulation of local species diversity in a stony, upland

    stream. *Ecological Monographs*, 68, 237-257.

Duong, L.M., Jeewon, R., Lumyong, S. and Hyde, K.D. 2006. DGGE coupled

    with ribosomal DNA gene phylogenies reveal uncharacterized fungal

    phylotypes. *Fungal Diversity*, 23, 121-138.

Elwood, J.W., Newbold, J.D., Trimble, A.F. and Stark, R.W. 1981. The limiting

    role of phosphorus in a woodland stream ecosystem: effects of P

    enrichment on leaf decomposition and primary producers. *Ecology*, 62,

    146-158.

Enriquez, S., Duarte, C.M. and Sand-Jensen, K. 1993. Patterns in decomposition

    rates among photosynthetic organisms: the importance of detritus C: N:

    P content. *Oecologia*, 94, 457-471.

Findlay, S. 2010. Stream microbial ecology. *Journal of the North American*

    Benthological Society, 29, 170-181.

Fisher, S.G. 1971. Annual energy budget of a small forest stream ecosystem:

    Bear Brook, West Thornton, New Hampshire, Ph.D. Thesis. Dartmouth

    College, Hanover, New Hampshire. 97 p.

Fisher, S.G. and Likens, G.E. 1973. Energy flow in Bear Brook, New Hampshire: an integrative approach to stream ecosystem metabolism. *Ecological Monographs*, 43, 421-439.

Gessner, M. 2005. Proximate lignin and cellulose. In: *Methods to study litter decomposition: a practical guide.* (Eds. F. Barlocher, Graca, M.A.S., Gessner, M.O.), pp. 115-120. Kluwer Academic Pub, Netherlands.

Gessner, M.O. 1997. Fungal biomass, production and sporulation associated with particulate organic matter in streams. *Limnetica*, 13, 33-44.

Gessner, M.O. and Chauvet, E. 1994. Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology*, 75, 1807-1817.

Gessner, M.O., Chauvet, E. and Dobson, M. 1999. A perspective on leaf litter breakdown in streams. *Oikos*, 85, 377-384.

Gore, J.A. 1985. Mechanisms of colonization and habitat enhancement for benthic macroinvertebrates in restored river channels. *The Restoration of Rivers and Streams: Theories and Experience* Ed. J.A. Gore, pp. 81-101. Butterworth Publishers, Boston, Massachusetts.

Graca, M. and Zimmer, M. 2005. Leaf toughness. In: *Methods to Study Litter Decomposition: a practical guide.* (Eds. F. Barlocher, Graca, M.A.S., Gessner, M.O.), pp. 121-125. Kluwer Academic Pub, Netherlands.

Graca, M.A.S. 2001. The role of invertebrates on leaf litter decomposition in streams: a review. *International Review of Hydrobiology*, 86, 383-393.

Gulis, V. and Suberkropp, K. 2003. Interactions between stream fungi and

bacteria associated with decomposing leaf litter at different levels of

nutrient availability. *Aquatic Microbial Ecology,* 30, 149-157.

Hagans, D.K., Weaver, W.E. and Madej, M.A. 1986. Long-term on-site and off-

site effects of logging and erosion in the Redwood Creek Basin, northern

California. p. 38-65 in Papers presented at the American Geophysical

Union meeting on cumulative effects. Technical bulletin 490. National

Council for Air and Stream Improvement, New York.

Harrison, E.T. 2007. The impact of fine sediment accumulation on benthic

macroinvertebrates: implications for river management. In: *Proceedings

of the 5th Australian Stream Management Conference.*

Helms, B. S., Schoonover J. E., and Feminella J. W. 2009. Seasonal variability of

landuse impacts on stream macroinvertebrate assemblages in streams

of western Georgia, USA. *Journal of the North American Benthological

Society*, 28, 991-1006.

Henley, W.F., Patterson, M.A., Neves, R.J. and Lemly, A.D. 2000. Effects of

sedimentation and turbidity on lotic food webs: a concise review for

natural resource managers. *Reviews in Fisheries Science*, 8, 125-139.

Hieber, M. and Gessner, M.O. 2002. Contribution of stream detrivores, fungi, and

bacteria to leaf breakdown based on biomass estimates. *Ecology*, 83,

1026-1038.

Houser, J.N., Mulholland, P.J. and Maloney, K.O. 2005. Catchment disturbance

and stream metabolism: patterns in ecosystem respiration and gross

primary production along a gradient of upland soil and vegetation disturbance. *Journal of the North American Benthological Society*, 24, 538-552.

Howarth, R.W. and Fisher, S.G. 1976. Carbon, nitrogen, and phosphorus dynamics during leaf decay in nutrient enriched stream microecosystems. *Freshwater Biology*, 6, 221-228.

Hullar, M.A.J., Kaplan, L.A. and Stahl, D.A. 2006. Recurring seasonal dynamics of microbial communities in stream habitats. *Applied and Environmental Microbiology*, 72, 713.

Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L. and Welch, D.M. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. *Genome Biology*, 8, R143.

Irons, J.G., Oswood, M.W., Stout, R. and Pringle, C.M. 1994. Latitudinal patterns in leaf litter breakdown: is temperature really important? *Freshwater Biology*, 32, 401-411.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M. and Sait, M. 2002. Improved Culturability of Soil Bacteria and Isolation in Pure Culture of Novel Members of the Divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Appl. Environ. Microbiol.,* 68, 2391-2396.

Kaushik, N.K. and Hynes, H.B.N. 1971. The fate of the dead leaves that fall into streams. *Archiv für Hydrobiologie*, 68, 465-515.

Lake, P.S. 2000. Disturbance, patchiness, and diversity in streams. *Journal of the North American Benthological Society*, 19, 573-592.

Larsson, U. and Hagstrom, A. 1979. Phytoplankton exudate release as an energy source for the growth of pelagic bacteria. *Marine Biology,* 52, 199-206.

Lear, G., Dong, Y. and Lewis, G. 2010. Comparison of methods for the extraction of DNA from stream epilithic biofilms. *Antonie van Leeuwenhoek*, 98, 567-571.

Lemly, A.D. 1982. Modification of benthic insect communities in polluted streams: combined effects of sedimentation and nutrient enrichment. *Hydrobiologia*, 87, 229-245.

Maier, R.M., Pepper, I.L. and Gerba, C.P. 2009. *Environmental microbiology*, Academic Press.

Maloney, D.C. and Lamberti, G.A. 1995. Rapid decomposition of summer-input leaves in a northern Michigan stream. *American Midland Naturalist*, 133, 184-195.

Maloney, K.O. and Weller, D.E. 2011. Anthropogenic disturbance and streams: land use and land-use change affect stream ecosystems via multiple pathways. *Freshwater Biology*, 56, 611-626.

Marmonier, P., Piscart, C., Sarriquet, P.E., Azam, D. and Chauvet, E. 2010. Relevance of large litter bag burial for the study of leaf breakdown in the hyporheic zone. *Hydrobiologia*, 641, 203-214.

McArthur, J.V. and Barnes, J.R. 1988. Community dynamics of leaf litter breakdown in a Utah alpine stream. *Journal of the North American Benthological Society*, 7, 37-43.

Melillo, J.M., Naiman, R.J., Aber, J.D. and Eshleman, K.N. 1983. The influence of substrate quality and stream size on wood decomposition dynamics. *Oecologia*, 58, 281-285.

Mille-Lindblom, C. and Tranvik, L.J. 2003. Antagonism between bacteria and fungi on decomposing aquatic plant litter. *Microbial Ecology,* 45, 173-182.

Minshall, G.W. 1967. Role of allochthonous detritus in the trophic structure of a woodland springbrook community. *Ecology*, 48, 139-149.

Moriarty, D.J.W. and Pullin, R.S.V. (1987) *Detritus and microbial ecology in aquaculture,* International Center for Living Aquatic Resources Management, Manilla, Philippines.

Naiman, R.J., Decamps, H. and Mcclain, M.E. 2005. *Riparia: ecology, conservation, and management of streamside communities*, Academic Press, Burlington, MA.

Newcombe, C.P. and Macdonald, D.D. 1991. Effects of suspended sediments on aquatic ecosystems. *North American Journal of Fisheries Management*, 11, 72 - 82.

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

Nykvist, N. 1961. Leaching and decomposition of litter. III. Experiments on leaf litter of *Betula verrucosa*. *Oikos*, 12, 249-263.

Nykvist, N. 1963. Leaching and decomposition of water-soluble organic substances from different types of leaf and needle litter. *Studio Forestalia Suecica*, 3, 1-29.

Palmer, M.A. and Poff, N.L. 1997. The influence of environmental heterogeneity on patterns and processes in streams. *Journal of the North American Benthological Society*, 16, 169-173.

Pereira, A.P., Graca, M.A.S., and Molles, M. 1998. Leaf litter decomposition in relation to litter physico-chemical properties, fungal biomass, arthropod colonization, and geographical origin of plant species. *Pedobiologia*, 42, 316-327.

Petersen, R.C. and Cummins, K.W. 1974. Leaf processing in a woodland stream. *Freshwater Biology*, 4, 343-368.

Porteous, L.A., Seidler, R.J. and Watrud, L.S. 1997. An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. *Molecular Ecology*, 6, 787-791.

Pusch, M., Fiebig, D., Brettar, I., Eisenmann, H., Ellis, B.K., Kaplan, L.A., Lock, M.A., Naegeli, M.W. and Traunspurger, W. 1998. The role of micro-organisms in the ecological connectivity of running waters. *Freshwater Biology*, 40, 453-495.

Rees, G.N., Watson, G.O., Baldwin, D.S. and Mitchell, A.M. 2006. Variability in sediment microbial communities in a semipermanent stream: impact of

drought. *Journal of the North American Benthological Society*. 25, 370-378.

Resh, V.H., Brown, A.V., Covich, A.P., Gurtz, M.E., Li, H.W., Minshall, G.W., Reice, S.R., Sheldon, A.L., Wallace, J.B. and Wissmar, R.C. 1988. The role of disturbance in stream ecology. *Journal of the North American Benthological Society*, 7, 433-455.

Riipinen, M.P., Davy Bowker, J. and Dobson, M. 2009. Comparison of structural and functional stream assessment methods to detect changes in riparian vegetation and water pH. *Freshwater Biology*, 54, 2127-2138.

Romani, A.M., Fischer, H., Mille-Lindblom, C. and Tranvik, L.J. 2006. Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. *Ecology*, 87, 2559-2569.

Roose-Amsaleg, C.L., Garnier-Sillam, E. and Harry, M. 2001. Extraction and purification of microbial DNA from soil and sediment samples. *Applied Soil Ecology*, 18, 47-60.

Ryan, P.A. 1991. Environmental effects of sediment on New Zealand streams: a review. *New Zealand Journal of Marine and Freshwater Research*, 25, 207-221.

Simon, K.S. 2000. Organic Matter Dynamics and Trophic Structure in Karst Groundwater. Ph.D. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Sinsabaugh, R.L., Antibus, R.K. and Linkins, A.E. 1991. An enzymic approach to the analysis of microbial activity during plant litter decomposition. Agriculture, *Ecosystems and Environment*, 34, 43-54.

Sinsabaugh, R.L. and Moorhead, D.L. 1994. Resource allocation to extracellular enzyme production: a model for nitrogen and phosphorus control of litter decomposition. *Soil Biology and Biochemistry*, 26, 1305-1311.

Sparrow, F.K. 1968. Ecology of freshwater fungi. In: *The fungal population, the fungi, an advanced treatise*. Eds G.C. Ainsworth and A.S. Sussman., pp. 41-93. Academic Press, New York.

Sponseller, R.A. and Benfield, E.F. 2001. Influences of land use on leaf breakdown in southern Appalachian headwater streams: a multiple-scale analysis. *Journal of the North American Benthological Society*, 20, 44-59.

Sponseller, R.A., Benfield, E.F. and Valett, H.M. 2001. Relationships between land use, spatial scale and stream macroinvertebrate communities. *Freshwater Biology*, 46, 1409-1424.

Stewart, B.A. 1992. The effect of invertebrates on leaf decomposition rates in two small woodland streams in southern Africa. *Archiv für Hydrobiologie*. Stuttgart, 124, 19-33.

Stout, R.J. 1989. Effects of condensed tannins of leaf processing in mid-latitude and tropical streams: a theoretical approach. *Canadian Journal of Fisheries and Aquatic Sciences*, 46, 1097-1106.

Suberkropp, K., Arsuffi, T.L. and Anderson, J.P. 1983. Comparison of degradative ability, enzymatic activity, and palatability of aquatic hyphomycetes grown on leaf litter. *Applied and Environmental Microbiology*, 46, 237-244.

Suberkropp, K. and Chauvet, E. 1995. Regulation of leaf breakdown by fungi in streams: influences of water chemistry. *Ecology*, 76, 1433-1445.

Suberkropp, K., Godshalk, G.L. and Klug, M.J. 1976. Changes in the chemical composition of leaves during processing in a woodland stream. *Ecology*, 57, 720-727.

Suberkropp, K. and Klug, M.J. 1976. Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology*, 57, 707-719.

Suberkropp, K. and Klug, M.J. 1980. The maceration of deciduous leaf litter by aquatic hyphomycetes. *Canadian Journal of Botany*, 58, 1025-1031.

Tate, C.M. and Gurtz, M.E. 1986. Comparison of mass loss, nutrients, and invertebrates associated with elm leaf litter decomposition in perennial and intermittent reaches of tallgrass prairie streams. *The Southwestern Naturalist*, 31, 511-520.

Taylor, B.R. and Barlocher, F. 1996. Variable effects of air-drying on leaching losses from tree leaf litter. *Hydrobiologia*, 325, 173-182.

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

Triska, F.J. 1970. Seasonal distribution of aquatic hyphomycetes in relation to the disappearnace of leaf litter form a woodland stream. Ph.D. Thesis, University of Pittsburgh.

U. S. Census Bureau. 2008.. *National Population Projects.* Retrieved June 8, 2011, from

http://www.census.gov/population/www/projections/2008projections.html.

Wallace, J.B. and Webster, J.R. 1996. The role of macroinvertebrates in stream ecosystem function. *Annual Review of Entomology*, 41, 115-139.

Wallace, J.B., Webster, J.R. and Cuffney, T.F. 1982. Stream detritus dynamics: regulation by invertebrate consumers. *Oecologia*, 53, 197-200.

Webster, J.R. and Benfield, E.F. 1986. Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics*, 17, 567-594.

Webster, J.R. and Waide, J.B. 1982. Effects of forest clearcutting on leaf breakdown in a southern Appalachian stream. *Freshwater biology*, 12, 331-344.

Weyers H. S. and Suberkropp, K. 1996. Fungal and bacterial production during the breakdown of yellow poplar leaves in 2 streams. *Journal of the North American Benthological Society* 15:408-420.

Widmer, F., Seidler, R.J., Donegan, K.K. and Reed, G.L. 1997. Quantification of transgenic plant marker gene persistence in the field. *Molecular Ecology*, 6, 1-7.

Widmer, F., Seidler, R.J. and Watrud, L.S. 1996. Sensitive detection of transgenic plant marker gene persistence in soil microcosms. *Molecular Ecology*, 5, 603-613.

Witkamp, M. 1963. Microbial populations of leaf litter in relation to environmental conditions and decomposition. *Ecology*, 44, 370-377.

Wommack, K.E., Bhavsar, J. and Ravel, J. 2008. Metagenomics: read length matters. *Applied and Environmental Microbiology,* 74, 1453.

Woods, P.V. and Raison, R.J. 1982. An appraisal of techniques for the study of litter decomposition in eucalypt forests. *Australian Journal of Ecology*, 7, 215-225.

Wright, M.S. and Covich, A.P. 2005. The effect of macroinvertebrate exclusion on leaf breakdown rates in a tropical headwater stream. *Biotropica*, 37, 403-408.

Young, R.G., Matthaei, C.D. and Townsend, C.R. 2008. Organic matter breakdown and ecosystem metabolism: functional indicators for assessing river ecosystem health. *Journal of the North American Benthological Society*, 27, 605-625.

1st stage:
leaching   24 hours

2nd stage:
conditioning   weeks

3rd stage:
fragmentation   months
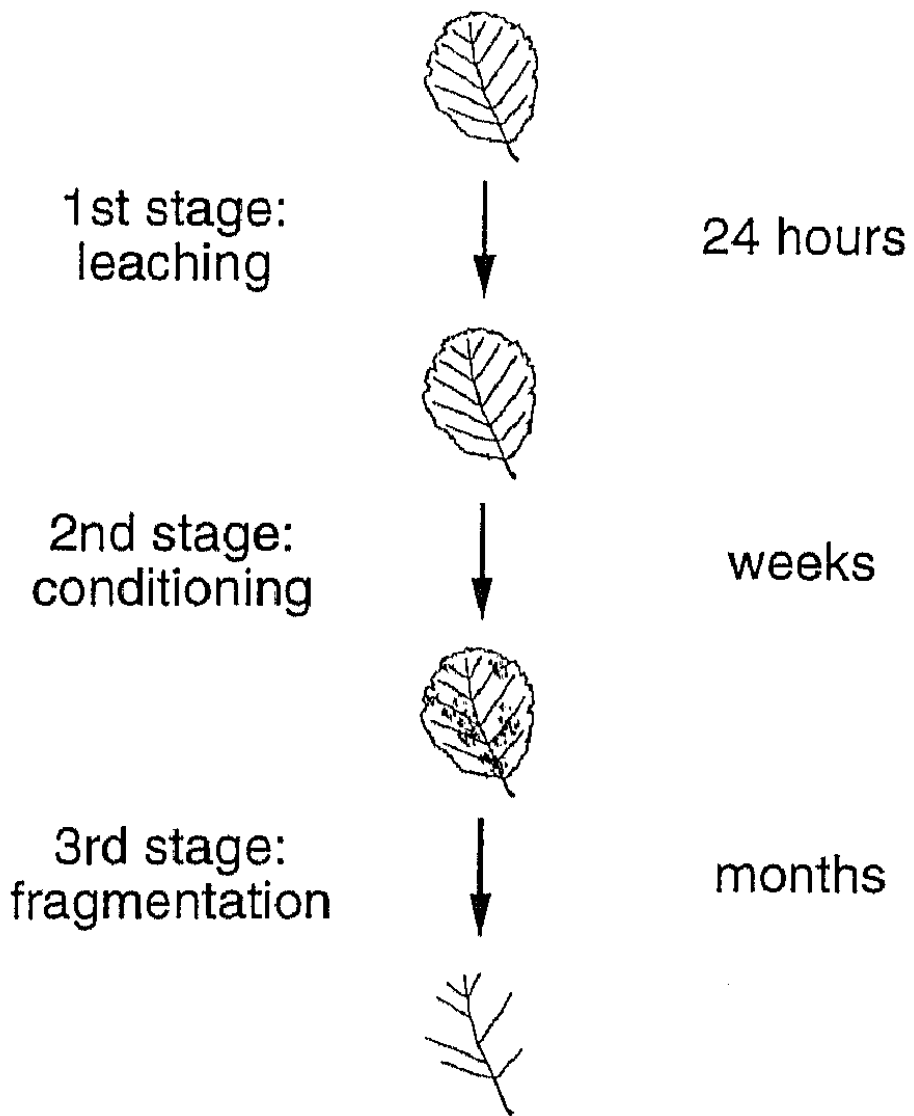
Figure 1.1. Stepwise process of instream leaf breakdown as proposed by Petersen and Cummins, 1974 (after Gessner, *et al.*, 1999).

A

Factor 1   Factor 2   Factor 3

Leaf mass loss

B

Shredder biomass   DOM

Fungal biomass   FPOM

Bacterial biomass CO_2, NH_4^+, PO_4^{3-}
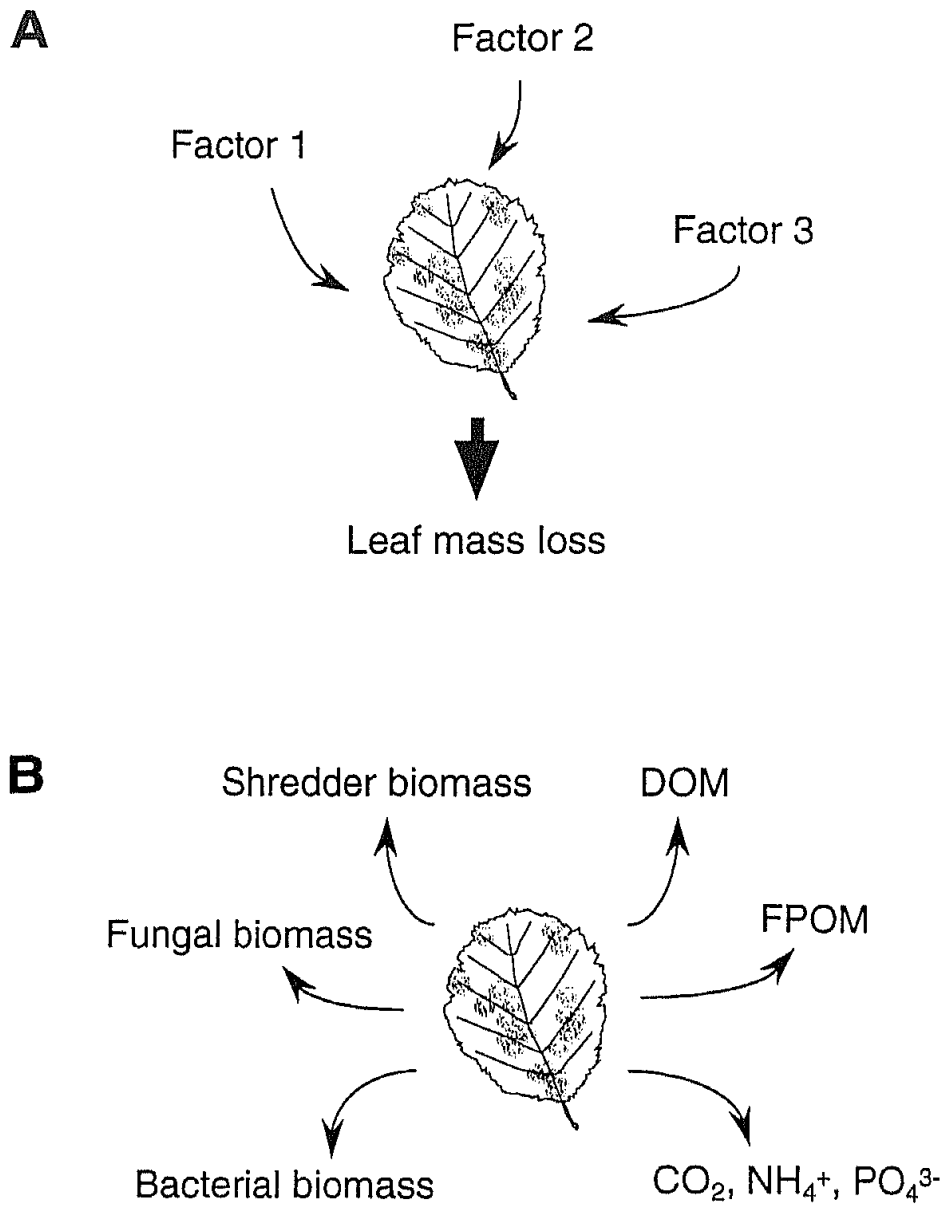
Figure 1.2. Contemporary (overlapping) approach to studying view of the processes driving leaf litter breakdown in streams, accounting for (a) factors acting on degrading leaf litter, and (b) primary and secondary products produced during breakdown (Gessner *et al.*, 1999). DOM, dissolved organic matter; FPOM, fine particulate organic matter.

41

CHAPTER II

PURIFICATION OF GENOMIC DNA EXTRACTED FROM ENVIRONMENTAL SOURCES FOR USE IN A POLYMERASE CHAIN REACTION

A. ABSTRACT

The ability to amplify genomic DNA in a polymerase chain reaction (PCR) is dependent upon the purity of the DNA template. Environmental genomic DNA often contains contaminants (e.g., polyphenols, humic acids, polysaccharides) that reduce template purity and can be difficult to remove, thereby inhibiting PCR amplification. There is thus a need for a method to purify extracted genomic DNA without reducing DNA concentration. In this protocol, extracted genomic DNA is embedded in agarose plugs and incubated in a formamide and salt (NaCl) solution to remove contaminants. The NaCl works to deproteinize and stabilize the DNA. The formamide serves to denature the DNA (which will subsequently be renatured within the agarose plug) and any contaminants that may be bound to the DNA. The purified DNA is extracted from the agarose plug using a standard commercial agarose extraction method, and the DNA may then be used as a template for PCR. Genomic DNA purified using this method has been shown to serve as an efficient template for PCR, without significant loss of DNA yield. An additional advantage of the method is that it allows the simultaneous processing of large numbers of samples at once.

B. INTRODUCTION

The increase in PCR efficiency using genomic DNA purified by this method is shown in Figure 2.1. A quantitative PCR was performed using a genomic DNA template purified by this method and the results were compared to those from templates prepared using a commercial kit and other treatment combinations. Liles *et al*. (2008) have described a method for purifying high-molecular-weight DNA for use in the construction of metagenomic libraries (see also **Isolation and Cloning of High-Molecular-Weight Metagenomic DNA from Soil Microorganisms** [Liles *et al*. 2009]). However, the protocol reported here is more rapid and is specifically designed for the purification of genomic DNA to be used as a template for PCR amplification.

C. RELATED INFORMATION

The increase in PCR efficiency using genomic DNA template purified using this protocol is shown in Figure 1. A quantitative PCR was performed using a genomic DNA template purified by this method and the results were compared to those from templates prepared using a commercial kit and other treatment combinations. Liles, *et al*. (2008) have described a method for purifying high-molecular-weight DNA for use in the construction of metagenomic libraries (see also Isolation and Cloning of High-Molecualr-Weight Metagenomic DNA from Soil Microorganisms [Liles *et al*., 2009]). However, the protocol reported here is more rapid and is specifically designed for the purification of

43

genomic DNA to be used as a template for PCR amplification.

D. PROTOCOL

1. Materials

1.1. Reagents

Agarose, molecular biology grade (Fisher) in 1x TAE

Conical tubes, 15 mL and 50 mL (sterile)

Formamide solution, stored at 4°C [R]

Glass beaker for making soft agarose (sterile)

Microcentrifuge tubes, 2 mL

TAE buffer stock, 50X [R]

1.2. Equipment:

Gel extraction kit (such as QIAquick Gel Extraction Kit or Wizard SV Gel and PCR Clean-Up System)

Graduated cylinder

Incubator, 15°C water bath

Laboratory microwave

Pipettes, p200 and p1000

Pipette tips, 200 µl and 1000 µl

Weigh boats

2. Method

2.1. Generation of agarose plugs and overnight incubation:

1. Extract genomic DNA from environmental sample.

   Genomic DNA may be directly extracted from an environmental sample using a bead beat lysis method (or commercial kit). Follow the published method for DNA extraction in terms of the amount of environmental sample to be processed. Typically, commercial kits will call for <500 mg of

environmental sample for extraction. Even with commercial kits that advertise "PCR-ready" DNA will be obtained, it is not unusual to have samples that may not be readily used as a PCR template. If after serial dilution of the DNA template no PCR product is obtained, and the positive controls have been successful, this protocol may be useful as a purification method to provide truly PCR-ready DNA.

2. Prepare an appropriate volume of 2% agarose solution in 1X TAE, heat in microwave, and let cool to 45°C.

   Mix briefly to produce a homogenous agarose plug. The purpose of embedding in agarose is to allow rapid DNA purification and subsequent washing of agarose plugs, without the need for DNA precipitation. Generally, 100 ml of 2% agarose is needed per sample if this is the volume of extracted DNA from the environmental sample.

3. Mix equal volumes of 2% agarose solution and an extracted DNA solution by pipetting briefly with a 1 mL pipet tip, in a 2 mL microcentrifuge tube.

   Here, 100 µL of 2% agarose was mixed by pipetting with 100 µL of extracted DNA solution. Embedding DNA into agarose allows for a matrix to contain DNA once it is denatured by the formamide/NaCl solution and allow contaminants to move into the surrounding liquid.

4. Allow agarose plug to solidify at room temperature in the microcentrifuge tubes directly.

   Less than 10 min should be required.

5. Add 5X volume of an 80% formamide/1.3M NaCl solution. Mix by slowly inverting in rack.

   Formamide serves as a denaturant to denature the DNA, and NaCl allows for stabilization of the DNA during incubation. If the total volume of the agarose plug is 200 ml (100 ml of 2% agarose and 100 ml of extracted DNA solution) then add a volume of 1 ml of formamide/salt solution.

6. Incubate samples for 1 hour at 15°C.

2.2. Washing of agarose plugs and extraction of DNA from agarose

7. Remove formamide and salt solution.

8. Wash 5X using 1mL 1X TAE.

   Add 1X TAE, invert in rack to mix, pipette off solution, and repeat.

9. Extract DNA from agarose.

   Effective kits for DNA removal include Qiagen's QIAquick Gel Extraction Kit or Promega's Wizard SV Gel and PCR Clean-Up System.

10. Gel-extracted DNA may now be used for subsequent procedures.

    DNA may be stored at -20°C or -85°C until further use.

E. DISCUSSION

Several methods exist for the purification of environmental DNA, including those that use phenolchloroform, hexadecyltrimethylammonium bromide (CTAB), polyvinylpolypyrrolidone (PVPP), cesium chloride density centrifugation, and hydroxyapatite column chromatographic purification (for reviews, see Roose-Amsaleg *et al*., 2001; Robe *et al*., 2003; see also Ogram *et al*., 1987; Holben *et al*., 1988; Knaebel & Crawford 1995). Steffan *et al.* (1988) have demonstrated that many of these methods (such as those using PVPP, cesium chloride, and hydroxyapatite) lower DNA yield. Often, a combination of two or more purification methods (such as a phenol-chloroform extraction followed by use of CTAB) is required to attain adequate purification of environmental DNA. However, using numerous purification steps not only can decrease DNA yield, but also increases sample-processing time. The latter is particularly disadvantageous when working with large numbers of samples. The protocol described here allows multiple samples to be processed at once.

The purification and amplification of environmental DNA can often be difficult because of low yields and co-isolation of contaminants. Incubation of genomic DNA in agarose plugs during formamide and salt treatment allows

removal of contaminants without significant loss of DNA. The protocol described here has been shown to be effective in purifying DNA from various environmental sources, such as soils, leaf litter, and marine corals, which have never successfully provided templates for PCR amplification. Commercial kits for genomic DNA extraction typically shear the DNA, resulting in fragment sizes <20 kb. The DNA yield obtained using this procedure will vary greatly depending on the initial DNA concentration. After following this procedure, each of the purified DNAs yielded abundant amplicons using ~20 ng of purified DNA as a template for PCR.

F. TROUBLESHOOTING

Problem: The agarose plug remains stuck in the bottom of the microcentrifuge tube. [Step 4]

Solution: It may be necessary to release the agarose plug from the bottom of the microcentrifuge tube by pressing the pipette tip down the side of the agarose plug. This step will insure total suspension of the agarose plug in the formamide and NaCl solution.

Problem: Insufficient purity of DNA.

[Step 5]

Solution: Depending upon the degree of contamination, the incubation time may be extended to overnight incubation at 15°C. Removal of the formamide and NaCl solution and replacing with fresh solution would be advisable with highly contaminated samples (i.e., having a change in color from phenolic or humic

47

compounds).

Problem: Insufficient yield of DNA.

[Step 8]

Solution: Reducing the volume of the elution buffer (to approximately 30 ml), as well as passing the elution buffer over the column two or more times, can help to maximize the yield of DNA recovered from the column. This is the only significant loss of DNA during the protocol, follow manufacturer's recommendations in DNA recovery from the agarose gel.

G. LITERATURE CITED

Holben, W.E., Jansson, J.K., Chelm, B.K., and Tiedje, J.M. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Appl Envirnon Microbiol* **54**:703-711.

Knaebel, D.B. and R.L. Crawford. 1995. Extraction and purification of microbial DNA from petroleum-contaminated soils and detection of low numbers of toluene, octane and pesticide degraders by multiple polymerase chain reaction and southern analysis. *Mol Ecol* **4**:579-591.

Liles, M.R., Williamson, L.L., Rodbumrer, J., Torsvik, V., Goodman, R.M., and Handelsman, J. 2008. Recovery, purification, and cloning of high molecular weight DNA from soil microorganisms. *Appl Environ Microbiol* **74**: 3302-3305.

Ogram, A., Sayler, G.S., Barkay, T. 1987. The extraction and purification of microbial DNA from sediments. *J Microbiol Methods* **7**:57-66.

Steffan, R.J., Goksoyr, J., Bej, A.K., Atlas, R.M. 1988. Recovery of DNA from soils and sediments. *Appl Environ Microbiol* **54**: 2908-2915.
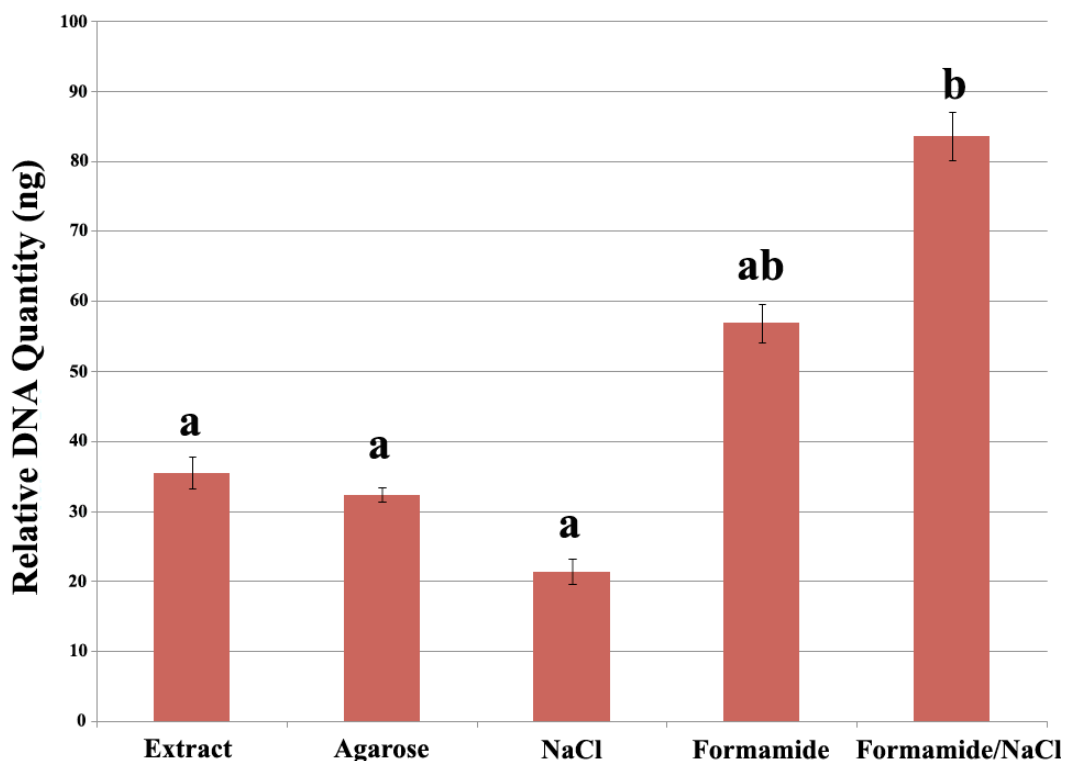
Figure 2.1. Bar graph representing amplified environmental genomic DNA

isolated from red maple (*Acer rubrum*) leaf litter. Genomic DNA was purified

using several treatment combinations and gel quantified prior to amplification. A

standardized amount of DNA (20ng) was then used in PCR reactions containing

a fluorescent probe (SYBR Green, Bio-Rad) that binds to double-stranded DNA,

and the fluorescence (R_n) was measured over time. Final DNA concentration was

then calculated by comparing fluorescence of a PCR standard of known DNA

concentration to the fluorescence of each sample. All points represent

standardized DNA quantity ± standard error. Letters indicate Tukey's multiple

comparison groupings.

CHAPTER III

EFFECTS OF BENTHIC MACROINVERTEBRATES ON MICROBIAL COMMUNITIES ASSOCIATED WITH LEAF BREAKDOWN IN SMALL COASTAL PLAINS STREAMS

A. ABSTRACT

Processing of allochthonous leaf litter within small, forested streams represents a valuable source of energy for stream food webs. Benthic macroinvertebrates and microorganisms play major separate but connected roles in leaf breakdown, with microorganisms colonizing and conditioning leaf litter, which allows for subsequent colonization and consumption by macroinvertebrates. The effect of macroinvertebrates on the leaf litter-associated microbial community was examined by conducting a 128-d *in situ* incubation using red maple and water oak leaves confined within coarse and fine mesh bags to control abundance of shredding macroinvertebrates. Samples were collected at various time points over 128 d to quantify leaf breakdown and characterize macroinvertebrate assemblages and microbial communities. Phospholipid fatty acid (PLFA) analysis was used to examine differences in fungal and bacterial biomass, and ribosomal intergenic spacer analysis (RISA) was used to assess changes in overall bacterial assemblage composition. Maple

breakdown significantly decreased in fine (vs. coarse) mesh treatments.

Identification of macroinvertebrates within leaf packs revealed ineffective

reduction of shredders due to a high abundance of smaller shredders including

Polypedilum and *Leuctra* species. No significant difference occurred between

coarse and fine mesh for microbial biomass estimates or RISA profiles. The

observed difference in maple breakdown between coarse and fine mesh leaf

packs may have occurred because of mesh-specific effects on the leaf pack

internal environment. Use of 1-mm mesh to reduce macroinvertebrate shredder

abundance may not be appropriate for all studies, depending on average

macroinvertebrate shredder size. Future studies should gauge the particular

shredders in the specific study streams and be sure to control for all sizes and

habits of shredders. In addition, studies should correct for mesh-specific leaf

mass loss by conducting mesh control studies under environmental conditions

similar to those experienced during the leaf breakdown study.

B. INTRODUCTION

Allochthonous inputs, particularly terrestrial leaf litter, represent a significant

portion of the available energy fueling heterotrophic, temperate-deciduous

forested streams (Fisher and Likens, 1973), but appears to play an insignificant

role in more autotrophic stream ecosystems (Minshall, 1978; Schade and Fisher,

1997). Instream breakdown of litter and its subsequent energy release to the

aquatic food web, integrates both physical (fragmentation) and biological

(nutrient mineralization) transformations, which involve the combined efforts of

microorganisms and macroinvertebrates (Webster and Benfield, 1986; Hieber and Gessner, 2002). Microorganisms play a major role in leaf breakdown, contributing up to 28% of overall mass loss (Hieber and Gessner, 2002).

Fungi tend to dominate during the initial phases of breakdown and decrease in biomass over time, contributing ~15% to overall mass loss (Kaushik and Hynes, 1971; Suberkropp and Klug, 1976; Hieber and Gessner, 2002). Although typically lower than fungi, bacterial biomass often increases as fungal biomass decreases and may contribute ~13% to overall leaf mass loss (Suberkropp and Klug, 1976; Weyers and Suberkropp 1996; Hieber and Gessner, 2002). Given their greater contribution during initial breakdown, studies have historically explored fungal colonization and succession on litter (Suberkropp and Klug, 1976; Nikolcheva and Barlocher, 2004); comparatively less work has been done to quantify the bacterial assemblage present during breakdown. Traditionally, studies have focused on culturable bacterial taxa present during breakdown (Suberkropp and Klug, 1976); however, more recent methodological advances allow for examination of bacterial assemblage composition without the previous culturing bias (Findlay, 2010).

By conditioning litter during breakdown, microorganisms also increase leaf palatability and quality for macroinvertebrates, which, in turn, can accelerate breakdown by fragmentation and consumption (Petersen and Cummins, 1974; Graca *et al.*, 1993; Wright and Covich, 2005). Fragmentation by macroinvertebrates can contribute up to 64% of leaf mass loss (Hieber and Gessner, 2002). Nutritional subsidies from microorganisms to macroinvertebrates

during breakdown have been recognized as being considerably higher than litter alone (Cummins, 1974; Graca, 2001). Macroinvertebrates preferentially consume and derive greater nutrition from, microbially conditioned leaf litter, but the effects macroinvertebrates have on microbial biomass and community composition on decomposing leaf litter have not been explored.

The purpose of this study was to assess the influence of macroinvertebrate presence on the litter-associated microbial community during breakdown, both as overall microbial biomass and bacterial assemblage composition. Specifically, this study was designed to examine 1) if bacterial and fungal biomass increased when abundances of large shredding macroinvertebrates were reduced, and 2) if bacterial assemblage composition differed between leaf packs where larger shredders were allowed to colonize and those where shredders were reduced.

C. METHODS

1. Study site

This study was conducted in Kings Mill Creek (UTM 0720701E 3600036N), a second-order, low-gradient stream at the Fort Benning Military Installation (FBMI) in west-central Georgia, USA. FBMI occurs south of the Fall Line in the Sand Hills subecoregion of the Southeastern Plains ecoregion (Griffith *et al.*, 2001). Kings Mill Creek is a clear (TSS = 5.45 mg/L), low-nutrient (NO3-N = 5.13 µg N/L; SRP = 6.04 µg/L), and acidic (pH = 4.33) stream with sandy substrate (Maloney *et al.*, 2005), and an intact deciduous riparian canopy consisting mostly of red maple (*Acer rubrum*), flowering dogwood (*Cornus florida*), yellow poplar

(*Liriodendron tulipifera*), sweetgum (*Liquidambar styraciflua*), sweetbay magnolia (*Magnolia virginiana*), black gum (*Nyssa sylcatica*), and water oak (*Quercus nigra*) (Cavalcanti, 2004). The Kings Mill Creek watershed was largely forested (85.6% cover, Maloney *et al.*, 2005) with a high abundance of shredder macroinvertebrates (K.O. Maloney and R. M. Mitchell, unpubl. data), implying the importance of litter to the stream's trophic economy.

2. Experimental design

An *in situ* litter decomposition experiment was conducted using artificial leaf packs of contrasting mesh sizes to control the abundance of large shredder macroinvertebrates colonizing and consuming leaf litter. *Acer rubrum* (red maple) and *Quercus nigra* (water oak) were used as leaf species for leaf pack construction. These species span a moderate range of breakdown rates, with red maple having a medium breakdown rate (k=0.005-0.010) and water oak a relatively slow rate (k<0.005) (Webster and Benfield, 1986). Both species were common in riparian zones at the study site and at FBMI in general (Cavalcanti, 2004; Lockaby *et al.*, 2005). There were 9 collection dates (days 0, 1, 2, 4, 8, 16, 32, 64, and 128) from January 4, 2007 to May 12, 2007, which included both initial microbial colonization and later periods of microbial succession as litter breakdown proceeded. Nine experimental blocks were established consisting of one run habitat per block containing 4 replicates of each leaf species x mesh size treatment, with each block sampled on 1 of the 9 dates; blocks were chosen randomly during the 128-d incubation.

Artificial leaf packs were held within nylon and fiberglass mesh bags (0.1524 m x 0.3048 m) placed *in situ*, a common method for studying leaf breakdown in streams (Boulton and Boon, 1991). Mesh bags of control leaf packs had coarse (6.35 mm) mesh on one side to allow colonization of macroinvertebrates and a smaller (3.175-mm) mesh on the opposite side to reduce loss of litter particles from inside the bag during incubation. In contrast, mesh bags of treatment leaf packs had a much finer mesh size (1-mm) on both sides composed of fiberglass window screening to exclude large macroinvertebrates. Control leaf packs were sewn closed with nylon, whereas the fine mesh leaf packs were closed using cable ties.

Leaves were collected weekly from a single tree of each species during fall 2006 (December-January) using tarps strung below trees to accumulate abscissed leaves. Leaves were then air-dried in a sterile Class II biosafety cabinet to a constant mass, weighed into 4-g packs (as dry mass), and then placed into sterilized mesh bags until deployed in the stream. A separate set of packs was sampled for each leaf species x mesh size treatment on day 0 by briefly dipping packs into stream water and then removing and returning them to the laboratory to quantify handling loss (Petersen and Cummins, 1974). Day 0 packs were considered to represent the initial phyllosphere (terrestrial) microbial community for each leaf species. On the specified collection date, each leaf pack was removed from the block, placed in a Ziploc bag, and returned on ice to the laboratory. A 2-leaf subsample was then collected from each leaf pack for microbial processing, and the remaining leaves were used for determining

breakdown rate (below). The leaf subsample was ground in liquid N_2 and stored at $-80°C$ until processed for microbial community characterization (below). To determine breakdown rate, the remaining leaf sample was dried to a constant mass at 60^oC, weighed, and then combusted the sample in a muffle furnace at 550^oC for 2 h. Once ashed, the residue was reweighed and subtracted from the pre-combusted dry mass for determination of ash-free dry mass (AFDM). Breakdown rates were calculated using an exponential decay model (Petersen and Cummins, 19974) as the slope of the regression line of ln(% AFDM remaining) vs time.

Sampled macroinvertebrates were stored in 70% ethanol and then sorted, measured for length (nearest mm), and identified to genus when possible, except for Oligochaeta and Acari (identified to subclass). Given their major role in litter processing, shredding macroinvertebrates were identified according to Merritt and Cummins (1996). Mean macroinvertebrate and shredder density (as number/g AFDM remaining) were estimated for each leaf species x mesh size treatment on each date. Litter pack macroinvertebrate assemblage composition was examined by estimating the percentage of taxa in the family Chironomidae and the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) as well as by estimating total taxon richness and Shannon diversity (*H'*). Total biomass of macroinvertebrates was calculated by converting body length into AFDM using published body-length dry-mass relationships for each leaf species x mesh size treatment (Benke *et al.*, 1999).

To characterize variation in physicochemical conditions known to affect breakdown (Webster and Benfield 1986; Dangles *et al.,* 2004) streamwater temperature, depth, and current velocity also were quantified. Water temperature was measured hourly with HOBO Temp data loggers secured to instream tree roots or submerged coarse woody debris. Current velocity differences lead to differential litter breakdown (Webster and Benfield, 1986) and also have been shown to affect microbial community establishment (e.g. initial biofilm formation) (Battin *et al.,* 2003). Streamwater depth also can alter leaf breakdown through its effects on water temperature, dissolved oxygen, and macroinvertebrate habitat (Webster and Benfield, 1986), and these effects on the instream environment have the potential to alter litter microbial communities. Because of these potential effects, differences in depth and current velocity were quantified initially and immediately before removal for each leaf pack to assess variation in leaf breakdown and microbial communities on a given sampling date attributable to depth or velocity differences. These values were compared between leaf species treatments to ensure that any observed differences between leaf species were not due to differences in depth or current velocity regime. Depth and current velocity measures were also used as covariates when comparing AFDM remaining and macroinvertebrate metrics between leaf species. Leaf pack depth was measured using a meter stick placed at the top center of each leaf pack, and a Marsh-McBirney Flowmate current meter was used to measure flow within each leaf pack. Mesh size differences could potentially alter current velocity, which, in turn, has the potential to alter the microbial community within a leaf

pack, so actual velocity conditions inside each leaf pack were simulated by positioning an empty "dummy" bag over the probe placed immediately upstream of each leaf pack prior to measurement.

3. Microbial lipids and community characterization

3.1 Microbial lipids. Phospholipid fatty acid (PLFA) analysis was used to quantify relative abundance of different lipids associated with bacteria and fungi (Zelles, 1999) on litter over the experiment using the following procedure. PLFA was adapted from Sasser (1990) for saponification, formation of fatty acid methyl esters (FAMEs), extraction, and a base wash. First, ~680-mg sample of the liquid N_2-ground litter was placed in a 20-mL test tube. Samples were saponified to liberate fatty acids from lipids of lysed cells with 1.0 mL saponification reagent (45 g NaOH, 150 mL methanol, 150 mL deionized water), vortexed for 10 s, heated to 100°C for 5 min in a boiling water bath, and then vortexed and reheated to 100°C for 25 min. FAMEs were formed through methylation by adding 2 mL of methylating reagent (325 mL 6.0 N HCl, 275 mL methanol), and vortexing and heating them to 80°C for 10 min. FAMEs were extracted from the aqueous phase into an organic phase using 1.25 mL extraction reagent (100 mL hexane, 100 mL methyl-tert butyl ether) tumbled for 10 min. Last, the aqueous phase was removed with a Pasteur pipette, washed samples with 3 mL of base wash (10.8 g NaOH, 900 mL distilled water) and tumbled for 5 min. Prior to chromatographic analysis, the organic phase containing FAMEs was transferred to glass vials, and FAMEs were analyzed using the Microbial Identification System (MIDI, Inc., Newark, DE, USA). The output provided known fatty acid

(FA) peak responses for fungi and bacteria from each sample, which were then used to estimate relative abundance of bacterial and fungal lipids. Based on FA data, relative fungal and bacterial lipid abundance was estimated and used to determine microbial lipid differences between species over the study. Relative abundance of bacteria was estimated using branched-chain saturated (e.g. *iso* and *anteiso*), hydroxyl (OH), monounsaturated, and cyclopropyl FAs (Zelles, 1999). Fungal relative abundance was estimated using 3 lipid markers (18:2ω6,9c, 18:1ω9c, and 18:3ω6c) (Guckert *et al*., 1985; Vestal and White, 1989).

3.2 DNA extraction for molecular analyses. Leaf subsamples were used for molecular analyses of bacterial communities via ribosomal intergenic spacer analysis (RISA). For this procedure, genomic DNA was isolated from 0.10-g leaf litter using a Qiagen genomic DNA extraction kit (Qiagen, Valencia, CA, USA). DNA was purified using a cetyltrimethylammonium bromide (CTAB) extraction procedure (Ausubel, 1994). In some cases, particularly for leaves at day 0, extracted DNA was not sufficiently pure to serve as template for PCR. For those samples, an additional round of genomic DNA purification was conducted using a combination 80% formamide and 1M NaCl treatment to provide PCR-ready genomic DNA template (see Chapter II). This formamide purification step has been tested with DNA extracted from many different environments and has not been observed to result in any loss of DNA or corresponding loss of diversity as assessed by denaturing gradient gel electrophoresis (DGGE). If this method was deemed necessary by the lack of PCR amplification using DNA templates

derived from commercial kit extraction, then the formamide purification method was applied consistently to all samples from that date.

3.3 RISA of ITS regions. Ribosomal Intergenic Spacer Analysis (RISA) was conducted by PCR amplification of bacterial internal transcribed spacer (ITS) regions and separation of polymorphic ITS amplicons within a polyacrylamide gel matrix. PCR was conducted within a volume of 10 μL containing GoGreen Master Mix (Promega; Madison, WI), 1x Bovine Serum Albumin (BSA), nuclease free water, primers, and approx. 1-5 ng genomic DNA template, quantified spectrophotometrically with a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Primers used for these reactions were the universal bacterial primers IRDYE 800-labeled ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') (Cardinale *et al.*, 2004) and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al.*, 2004) at a final concentration of 0.20 μM. This primer set has been shown to be less susceptible as other primers to known PCR biases such as those from substrate reannealing (Suzuki and Giovannoni, 1996) and preferential amplification of shorter DNA templates (Cardinale *et al.*, 2004). Amplification was done according to the method of Fisher and Triplett (1999), as follows: reaction mixtures were held at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 15 s, 55°C for 15 s, and 72°C for 45 s, and a final extension of 72°C for 2 min. PCR products were verified on a 1% agarose gel stained with ethidium bromide. Following verification of product yield and size, amplicons were separated in a 5.5% polyacrylamide gel matrix and images were recorded using a Li-Cor 4300 (Li-Cor Inc., Lincoln, NE, USA). Bands were defined relative to the

highest band density for that pattern; all bands with a density >10% of the highest band density were used to create a presence-absence matrix for further analysis.

4. Mesh control study

To determine whether or not observed litter breakdown differences were due to mesh-specific effects on breakdown rather than actual reduced macroinvertebrate abundance, leaf breakdown was quantified in both mesh sizes in the absence of macroinvertebrates. In May 2011 a mesh-control study was conducted in an indoor artificial stream (84 in. x 14 in. x 10.75 in.). Five gram maple and oak leaf packs were constructed and contained in both fine and coarse mesh bags, with 4 replicates of each leaf x mesh treatment arrayed in a Latin square design. Packs were incubated for 32 d exposed to a continuous flow of water from a nearby pond. Current velocity (mean = 0.09 m/s, for both species) within the channels was controlled to mimic conditions experienced in the original study. After 32 d, leaf packs were removed, and AFDM remaining was calculated as previously mentioned. An additional set of packs from each treatment was used to estimate handling loss, processed as above.

5. Hypotheses and analyses

Our overall hypothesis was that a reduction in macroinvertebrate abundance, particularly shredders, would lead to a significant shift in litter-associated microbial community composition. Given the preferential feeding of macroinvertebrates on microbially conditioned leaves, we also hypothesized that bacterial biomass would be lower in leaf packs with macroinvertebrates present than in leaf packs without macroinvertebrates. With regard to leaf breakdown, we

hypothesized that both leaf species would have a slower breakdown rate under reduced macroinvertebrate abundance.

A general linear model, including days in stream, mesh treatment, leaf species and all possible interactions, using depth as a covariate, was used to test for differences in relative abundance of bacterial and fungal biomass and depth based on mesh and leaf species treatments. Tukey's multiple comparison tests (Zar, 1999) were used to compare bacterial and fungal biomass among leaf species x mesh treatments for a given time point. Relative abundance of bacterial and fungal biomass, as well as depth, was square root transformed to achieve normality. Current velocity did not follow a normal distribution and was compared between mesh treatments and among days in stream using a Kruskal-Wallis test. AFDM remaining in our initial and mesh control studies was compared between mesh treatments for each leaf species using a one-way ANOVA. AFDM remaining values in our initial study were arcsine transformed to obtain a normal distribution prior to analysis. Due to their non-normal distribution, a nonparametric Kruskal-Wallis test was used to compare macroinvertebrate metrics between coarse and fine mesh for each leaf species (Kruskal and Wallis, 1952).

Bray-Curtis dissimilarity matrices (Bray and Curtis, 1957) were calculated and used to compare microbial community (FAME profiles) and bacterial assemblage (RISA profiles) composition between leaf species and mesh treatments. In order to examine natural groupings among samples, cluster analysis was conducted to help visualize differences in bacterial assemblage composition from RISA

profiles. A hierarchical, average linkage method was used because of its ability to be less influenced by extreme dissimilarity values, and Bray-Curtis was used because it is not influenced by joint absences of species in two samples (Bray and Curtis, 1957). An Analysis of Similarity (ANOSIM) (Clarke, 1993) was used to test whether observed separation among treatment groups based on FAME and RISA profiles was significant. ANOSIM is equivalent to a 1-way ANOVA based on multispecies data and produces a global R value that ranges from -1 to +1 with values greater than 0 indicating greater dissimilarity between treatments than among samples (Chapman and Underwood, 1999). Global R values near 0 indicate no significant difference between treatments. For all statistical tests α=0.05.

D. RESULTS

Water temperature during the 128-d incubation (January to May) ranged from 3.7 to 30.3°C, with a mean of 13.4°C. Current velocity immediately upstream of leaf packs did not differ significantly over time (p=0.236) or between mesh treatments (p=0.584) (Table 3.1). Depth of leaf packs varied significantly over the study (p<0.001); however, mean depth overall did not differ between mesh treatments (p=0.949) (Table 3.1) and was not a significant covariate (p=0.415) in explaining AFDM remaining throughout this study.

Overall, in coarse-mesh packs, maple leaves (k=0.017) degraded ~2.8x faster than oak (k=0.006). Whereas, in fine-mesh packs, differences between oak and maple were less pronounced (Table 3.1). Regarding mesh treatment, AFDM

remaining for maple leaf packs was significantly lower (breakdown was faster) for coarse (vs. fine) mesh ($p<0.001$; Fig. 3.1). Although breakdown of oak was slightly less in fine- (vs. coarse) mesh packs (k=0.005 and 0.006, respectively), there was no significant difference in AFDM remaining (p=0.159) (Table 3.1).

The mesh-size treatments were ineffective in producing differences in macroinvertebrate assemblages, as no significant differences existed between coarse and fine mesh packs of oak or maple for any macroinvertebrate metrics (Table 3.2). Chironomidae larvae dominated leaf packs of both species and mesh treatments (60-75% of total abundance, Table 3.2). Mean macroinvertebrate density ranged from ~24 to 51 individuals per g AFDM. Maple leaf packs had a higher diversity of macroinvertebrates than oak (p=0.038) and contained a significantly higher density of shredder macroinvertebrates than oak (p=0.005). The shredder functional feeding group present within maple leaf packs was dominated by *Polypedilum* sp. (Chironomidae) (~63%), *Leuctra* sp. (Leuctridae) (~34%), and to a lesser extent, oribatid mites (Acari: Oribatida) (~3%). Oak leaf packs contained similar relative abundances of shredders, being dominated by *Polypedilum* sp. (~61%), *Leuctra* sp. (~37%), and oribatid mites (~2%). Specifically looking at shredder biomass, the dominant contributor to shredder biomass in maple packs was *Tallaperla* sp. (~56%), followed by *Tipula* sp. (~19%), *Polypedilum* sp. (~14%), and *Leuctra* sp. (~8%). Shredder biomass in oak leaf packs consisted largely of *Polypedilum* sp. (~46%) and *Leuctra* sp. (~33%), and to a lesser extent *Tipula* sp. (~7%) and *Tallaperla* sp. (~6%). With regard to overall macroinvertebrate biomass, biomass consisted of larvae of the

family Chironomidae (~18%) and members of the families Odontoceridae (~18%) and Elmidae (~14%).

Overall comparison of FAME profiles to examine differences in microbial community structure indicated no significant difference in lipid profile composition between coarse and fine mesh, either for maple (Global R = 0.02, p=0.236) or oak (Global R=-0.016, p=0.563). However, there was a significant difference in lipid profile composition between leaf species (Global R=0.47, p=0.001). Comparison of bacterial and fungal biomass estimates using the selected bacterial- and fungal-associated lipid markers revealed no difference between coarse and fine mesh packs (p=0.880 and 0.675, respectively) (Figs. 3.2 and 3.3). However, there was a significant difference between maple and oak in both bacterial and fungal biomass with oak having more fungal biomass and maple more bacterial biomass (p<0.001) (Figs. 3.2 and 3.3). Both bacterial and fungal biomass varied significantly with incubation time (p=0.007 and <0.001, respectively). The most common bacterial lipids were 15:1w6c, 15:0 ISO, cy19:0, and cy17:0, and the most common fungal lipid was 18:2w6,9c.

RISA results indicated no difference in the bacterial assemblage composition between coarse and fine mesh overall (Global R=0.006, p=0.250). However, bacterial assemblage composition differed between leaf species (Global R=0.038, p=0.03). When separated by leaf species, no significant difference was observed between coarse and fine mesh for maple (Global R=0.026, p=0.108) or oak (Global R=0.023, p=0.128) separately (Figs. 3.4 and 3.5). Bacterial

assemblage composition also varied significantly over time (Global R=0.464, *p*=0.001).

Mean current velocity was 0.09 m/s for both maple and oak leaf species during the mesh-control study conducted in artificial streams, and did not differ between coarse and fine mesh for maple or oak (*p*=0.883 and 0.206, respectively). After 32 d, maple in coarse mesh bags had 26.58% (± 1.01% SE) AFDM remaining vs. 30.08% (± 1.80% SE) for fine mesh bags. Oak in coarse mesh contained 71.98% (± 0.72% SE) AFDM remaining compared to 74.75% (± 0.83% SE) AFDM remaining in fine mesh leaf packs. Although fine mesh leaf packs contained a larger amount of AFDM remaining than coarse mesh leaf packs overall, the difference between mesh types was only slightly significant for oak (F=6.34, *p*=0.045) and not maple (F=2.33, *p*=0.178).

E. DISCUSSION

Based on abundance, richness, and biomass data, the intended reduction of macroinvertebrates in fine- vs. coarse-mesh bags, especially for large shredders, was not achieved. This result was surprising given the widespread and effective use of fine (1mm) mesh in macroinvertebrate exclusion studies of leaf breakdown (O'Connor *et al*., 2000; Wright and Covich, 2005). Many studies using this mesh size, however, have been attempting to exclude larger shredders (e.g. freshwater shrimps) (Hein and Crowl, 2010). The most commonly found shredders observed in our study were *Polypedilum* larvae and nymphs of the stonefly *Leuctra*, many of which were ~1mm in length and apparently were capable of passing through

the fine-mesh bags. The exclusion (or reduction) of macroinvertebrates in this study was not effective. So, we conclude that there was no difference in the microbial community, as biomass or bacterial assemblage composition between coarse and fine mesh treatments, likely because there was no significant reduction in macroinvertebrates. Given that the exclusion was not effective, a possible effect of macroinvertebrates on the microbial community cannot be ruled out. In order to circumvent this uncertainty, an additional study, possibly one using a mesh size <1mm, would need to be conducted that ensured an effective exclusion of macroinvertebrate shredders and other groups.

The observed mesh effect on maple leaf breakdown rate could have potentially been due to an altered internal environment of fine mesh leaf packs given the slight increase in AFDM remaining in fine mesh packs during our mesh control study. AFDM remaining was not significantly different between coarse and fine mesh in our control study, although this was likely due to variability in AFDM remaining for maple leaves during the warmer season in which the control study was conducted (May vs. January). If this mesh control study were repeated during the same time period (and temperature regime) that our initial study was conducted, we may have been more likely to see a greater effect of mesh size on AFDM remaining for maple leaves. Future studies attempting to exclude macroinvertebrate shredders of a length similar to that observed in this study and using 1-mm mesh would need to quantify mesh size-specific effects during the same season that their study is conducted and correct for this when calculating AFDM remaining.

Aside from the inability to effectively test macroinvertebrate-feeding effects on the leaf litter-associated microbial community in this study, we did observe significant leaf species differences in the microbial community, both in overall biomass as well as bacterial assemblage composition. Others have noted effects of leaf species on overall microbial activity and biomass (Gulis and Suberkropp, 2003). Blair *et al.* (1990) attributed differences in N fluxes in mixed- and single-species leaf packs to differences in the decomposer community, including microbial density, again suggesting the ability of leaf species to alter microbial community biomass and/or composition. In this study, estimates of microbial biomass and bacterial assemblage composition varied between maple and oak leaves. These leaf species also showed significant differences in macroinvertebrate diversity and shredder density, as well as rate of breakdown, with maple leaves having higher macroinvertebrate diversity, shredder density, and a faster breakdown rate. It is possible that the observed leaf species differences in microbial community structure are due to combined differences in the leaf litter macroinvertebrate assemblage (e.g. diversity, density), as well as differences in leaf chemistry (e.g. N or lignin content).

The results of our study indicate that use of 1-mm mesh to exclude macroinvertebrate shredders is not an effective practice in streams where many macroinvertebrate shredder taxa are fairly short in length (<5mm) and/or slender bodied, such as in our study. In addition, our results highlight the potential of leaf species differences to alter macroinvertebrate assemblage composition, as well as microbial community biomass and composition during leaf breakdown in

streams. In order to tease apart whether the differences in microbial community composition observed in this study were due to leaf chemistry differences or macroinvertebrate assemblage composition, an effective macroinvertebrate exclusion would need to be accomplished and would likely require use of a much finer (<1mm) mesh.

F. LITERATURE CITED

Ausubel, F.M. 1994. Preparation of plant DNA using CTAB. *Current Protocols in Molecular Biology,* Suppl 27, 2.3-2.3.7.

Battin, T.J., Kaplan, L.A., Newbold, J.D., Cheng, X. and Hansen, C. 2003. Effects of Current Velocity on the Nascent Architecture of Stream Microbial Biofilms. *Applied and Environmental Microbiology,* 69, 5443-5452.

Benke, A.C., Huryn, A.D., Smock, L.A. and Wallace, J.B. 1999. Length-mass relationships for freshwater macroinvertebrates in North America with particular reference to the southeastern United States. *Journal of the North American Benthological Society*, 308-343.

Blair, J.M., Parmelee, R.W. and Beare, M.H. 1990. Decay rates, nitrogen fluxes, and decomposer communities of single-and mixed-species foliar litter. *Ecology,* 71, 1976-1985.

Boulton, A.J. and Boon, P.I. 1991. A review of methodology used to measure leaf litter decomposition in lotic environments: time to turn over an old leaf? *Marine and Freshwater Research,* 42, 1-43.

Bray, J.R. and Curtis, J.T. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs,* 27, 325-349.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., Zanardini, E., Sorlini, C., Corselli, C. and Daffonchio, D. 2004. Comparison of Different Primer Sets for Use in Automated Ribosomal Intergenic Spacer Analysis of Complex Bacterial Communities. *Applied and Environmental Microbiology,* 70, 6147-6156.

Cavalcanti, G.G. 2004. Effects of sediment deposition in aboveground net primary productivity, vegetation composition, structure, and fine root dynamics in riparian forests. *MS Auburn University, Auburn, AL*.

Chapman, M.G. and Underwood, A.J. 1999. Ecological patterns in multivariate assemblages: information and interpretation of negative values in ANOSIM tests. *Marine ecology. Progress series,* 180, 257-265.

Clarke, K. R. 1993. Non-parametric multivariate analysis of changes in community structure. *Australian Journal of Ecology* 18, 117-143.

Cummins, K.W. 1974. Structure and function of stream ecosystems. *BioScience,* 24, 631-641.

Dangles, O., Gessner, M.O., Guerold, F. and Chauvet, E. 2004. Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. *Journal of Applied Ecology,* 41, 365-378.

Findlay, S. 2010. Stream microbial ecology. *Journal of the North American Benthological Society*, 29, 170-181.

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

Fisher, S.G. and Likens, G.E. 1973. Energy flow in Bear Brook, New Hampshire: an integrative approach to stream ecosystem metabolism. *Ecological Monographs,* 43, 421-439.

Graca, M., Maltby, L. and Calow, P. 1993. Importance of fungi in the diet of
Gammarus pulex and Asellus aquaticus. *Oecologia,* 96, 304-309.

Graca, M.A.S. 2001. The role of invertebrates on leaf litter decomposition in
streamsña review. *International Review of Hydrobiology,* 86, 383-393.

Griffith, G.E., Omernik, J. M., Comstock, J. A., Lawrence, S., Martin, G.,
Goddard, A., Hulcher, V. J., Foster, T. 2001. Ecoregions of Alabama and
Georgia color poster with map, descriptive text, summary tables, and
photographs.. US Geological Survey, Reston, Virginia.

Guckert, J.B., Antworth, C.P., Nichols, P.D. and White, D.C. 1985. Phospholipid,
ester-linked fatty acid profiles as reproducible assays for changes in
prokaryotic community structure of estuarine sediments. *FEMS
Microbiology Letters,* 31, 147-158.

Gulis, V. and Suberkropp, K. 2003. Interactions between stream fungi and
bacteria associated with decomposing leaf litter at different levels of
nutrient availability. *Aquatic Microbial Ecology,* 30, 149-157.

Hein, C.L. and Crowl, T.A. 2010. Running the predator gauntlet: do freshwater
shrimp (Atya lanipes) migrate above waterfalls to avoid fish predation?
Journal of the North American Benthological Society, 29, 431-443.

Hieber, M. and Gessner, M.O. 2002. Contribution of stream detrivores, fungi, and
bacteria to leaf breakdown based on biomass estimates. *Ecology,* 83,
1026-1038.

Houser, J.N., Mulholland, P.J. and Maloney, K.O. 2005. Catchment disturbance
and stream metabolism: patterns in ecosystem respiration and gross

primary production along a gradient of upland soil and vegetation

disturbance. *Journal of the North American Benthological Society,* 24,

538-552.

Kaushik, N.K. and Hynes, H.B.N. 1971. The fate of the dead leaves that fall into

streams. *Archiv für Hydrobiologie*, 68, 465-515.

Kruskal, W.H. and Wallis, W.A. 1952. Use of ranks in one-criterion variance

analysis. *Journal of the American statistical Association***,** 47, 583-621.

Lockaby, B.G., Schilling, R., Cavalcanti, E. and Hartsfield, G. 2005. Effects of

sedimentation on soil nutrient dynamics in riparian forests. *Journal of

Environmental Quality,* 34, 390.

Maloney, K., Mulholland, P. and Feminella, J. 2005. Influence of Catchment-

Scale Military Land Use on Stream Physical and Organic Matter Variables

in Small Southeastern Plains Catchments USA.. *Environmental

Management,* 35, 677-691.

Maloney, K.O. and Feminella, J.W. 2006. Evaluation of single-and multi-metric

benthic macroinvertebrate indicators of catchment disturbance over time

at the Fort Benning Military Installation, Georgia, USA. *Ecological

Indicators,* 6, 469-484.

Merritt, R.W. and Cummins, K.W. 1996. (Editors) *An introduction to the aquatic

insects of North America, 2nd edition.* Kendall Hunt.

Minshall, G.W. 1978. Autotrophy in stream ecosystems. *BioScience*, 28, 767-

771.

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

O'connor, P.J., Covich, A.P., Scatena, F.N. and Loope, L.L. 2000. Non-indigenous bamboo along headwater streams of the Luquillo Mountains, Puerto Rico: leaf fall, aquatic leaf decay and patterns of invasion. *Journal of Tropical Ecology,* 16, 499-516.

Petersen, R.C. and Cummins, K.W. 1974. Leaf processing in a woodland stream. *Freshwater Biology,* 4, 343-368.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. In: *Technical Note #101.* Microbial ID, Newark, DE.

Schade, J.D. and Fisher, S.G. 1997. Leaf litter in a Sonoran Desert stream ecosystem. *Journal of the North American Benthological Society*, 16, 612-626.

Suberkropp, K. and Klug, M.J. 1976. Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology*, 57, 707-719.

Suzuki, M.T. and Giovannoni, S.J. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology,* 62, 625.

Vestal, J.R. and White, D.C. 1989. Lipid analysis in microbial ecology. *BioScience,* 39, 535-541.

Webster, J.R. and Benfield, E.F. 1986. Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics,* 17, 567-594.

Weyers H. S. and Suberkropp, K. 1996. Fungal and bacterial production during the breakdown of yellow poplar leaves in 2 streams. *Journal of the North American Benthological Society* 15:408-420.

Wright, M.S. and Covich, A.P. 2005. The effect of macroinvertebrate exclusion on leaf breakdown rates in a tropical headwater stream. *Biotropica,* 37, 403-408.

Zar, J.H. 1999. *Biostatistical analysis,* Prentice hall Upper Saddle River, NJ.

Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. *Biology and Fertility of Soils,* 29, 111-129.

Table 3.1. Mean (± 1SE) physicochemical variables and leaf litter breakdown rates for each mesh and leaf species treatment. Coarse = 6.38mm; Fine = 1mm.

Variable	Maple		Oak	
	Coarse	Fine	Coarse	Fine
Depth (m)	0.17± 0.01	0.16± 0.01	0.17 ± 0.01	0.18 ± 0.01
Current velocity (m/s)	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Breakdown rate (k)	0.017	0.008	0.006	0.005
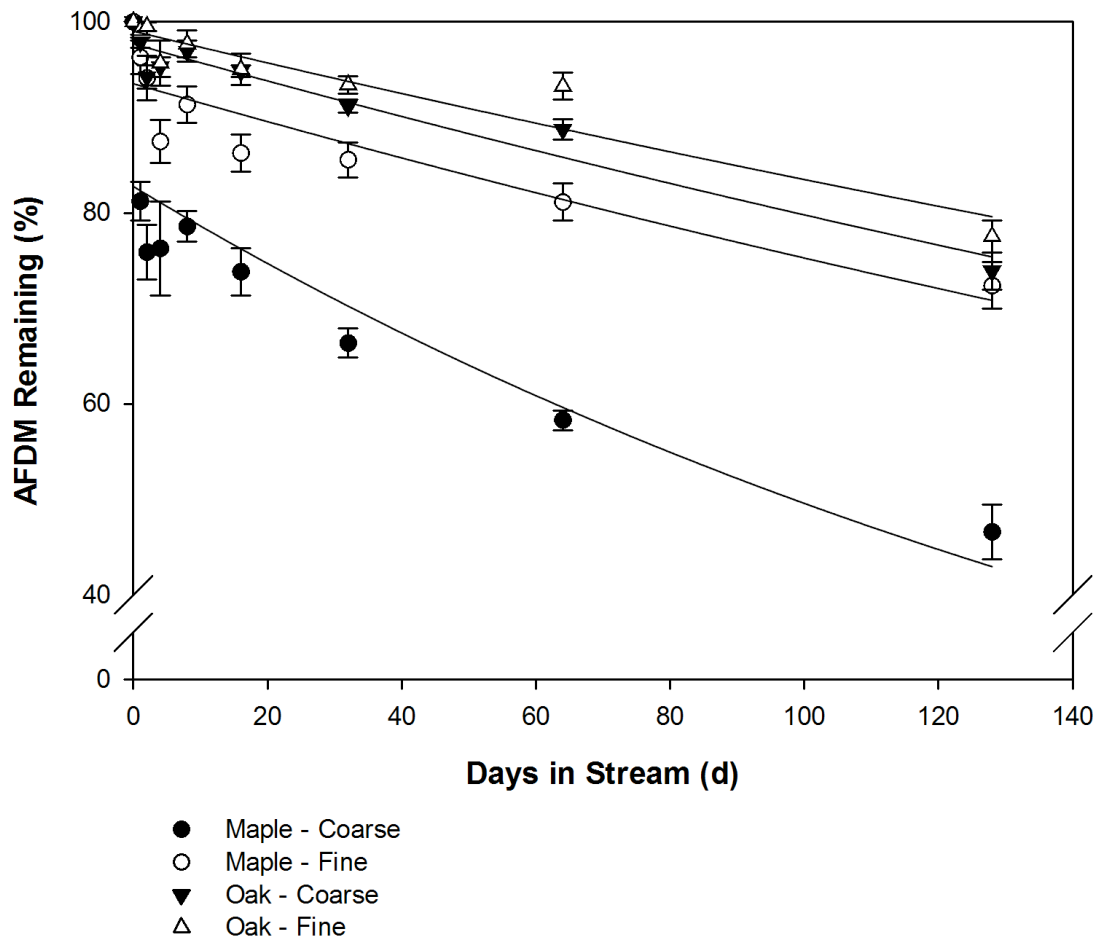
Figure 3.1. Ash-free dry mass (AFDM) remaining of red maple and water oak leaf packs confined within fine- and coarse-mesh treatments over a 128-d incubation in Kings Mill Creek, GA, USA. Plotted points are means (± 1SE).

78

Table 3.2. Benthic macroinvertebrate metrics (mean ± 1SE) for each leaf species and mesh treatment combination during a 128-d incubation in Kings Mill Creek, GA, USA. Coarse = 6.38mm; Fine = 1mm.

Metric	Maple			Oak		
	Coarse	Fine	p	Coarse	Fine	p
Macroinvertebrate abundance	53.52 ± 19.19	114.8 ± 35.21	0.052	63.94 ± 22.48	102.37 ± 31.86	0.224
Shredder abundance	17.90 ± 7.33	50.19 ± 17.64	0.093	20.94 ± 7.39	49.37 ± 18.83	0.314
Richness	7.52 ± 1.17	9.22 ± 1.12	0.106	6.69 ± 1.10	7.88 ± 1.22	0.399
Shannon diversity (H')	1.37 ± 0.13	1.54 ± 0.09	0.301	1.14 ± 0.15	1.24 ± 0.12	0.736
Biomass	11.08 ± 3.92	15.72 ± 5.16	0.485	8.37 ± 2.40	14.81 ± 7.64	0.979
Macroinvertebrate density (ind./g AFDM)	34.77 ± 13.45	51.17 ± 15.99	0.153	23.93 ± 8.80	35.37 ± 11.17	0.259
Shredder density (ind./g AFDM)	12.0 ± 5.11	22.34 ± 7.81	0.179	7.85 ± 2.89	16.82 ± 6.29	0.315
% Chironomidae	69.47 ± 5.56	74.86 ± 4.50	0.756	59.80 ± 6.39	68.76 ± 5.95	0.428
% EPT	9.31 ± 2.52	8.16 ± 1.99	0.554	13.87 ± 4.43	17.07 ± 4.84	0.925
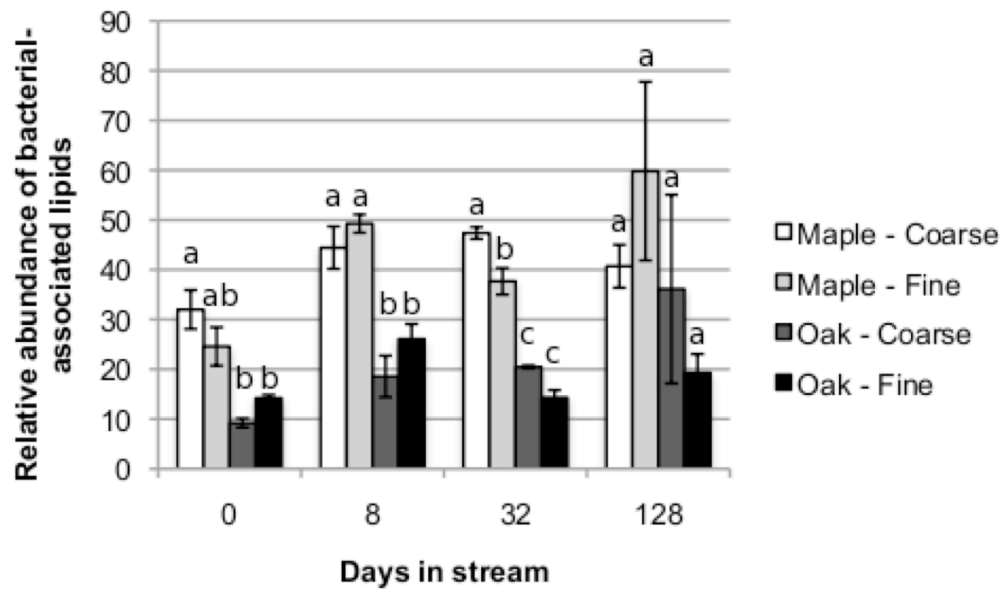
Figure 3.2. Relative abundance (mean % ± 1SE) of select bacterial lipid markers of red maple and water oak leaf packs over the 128-d incubation in Kings Mill Creek, GA, USA.
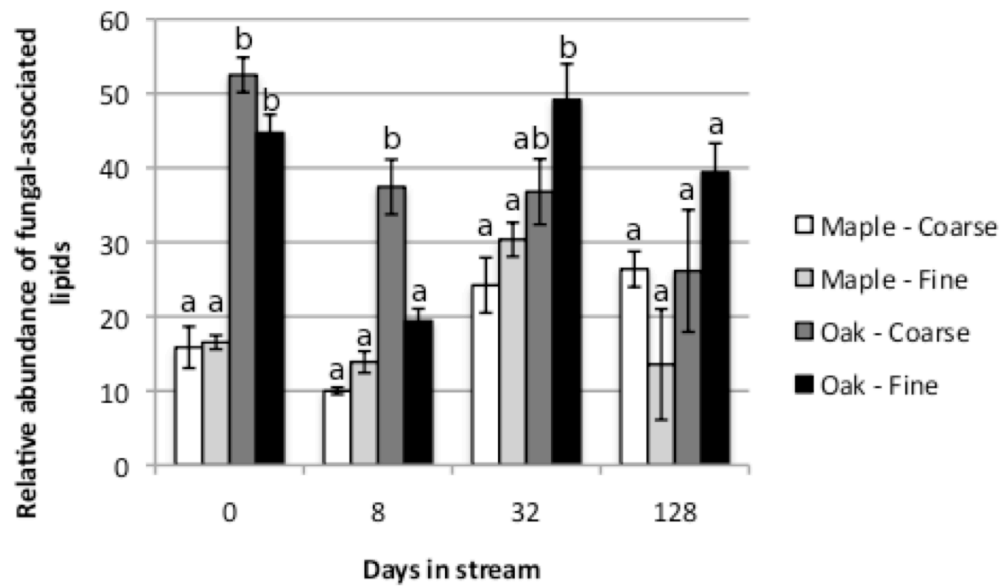
Figure 3.3. Relative abundance (mean % ± 1SE) of select fungal lipid markers of red maple and water oak leaf packs over the 128-d incubation in Kings Mill Creek, GA, USA.
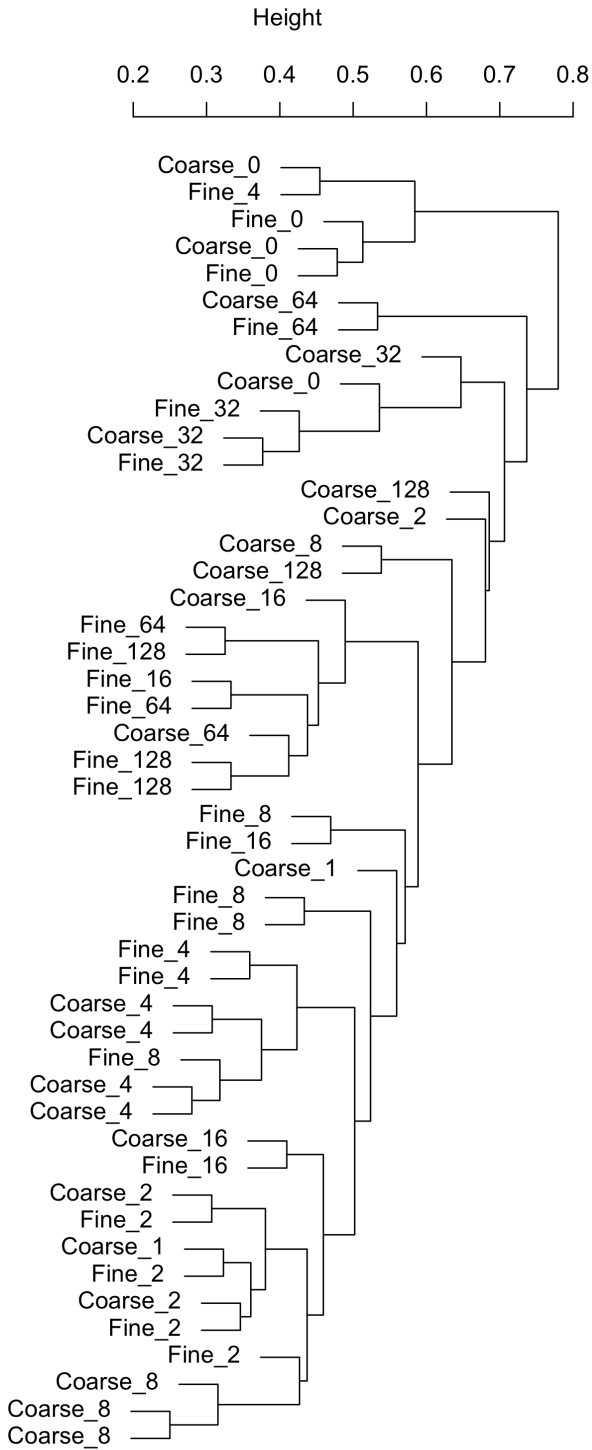
Figure 3.4. Average linkage dendrogram based on Bray-Curtis dissimilarity measures between red maple leaf pack bacterial assemblage RISA profiles of coarse and fine mesh treatments.
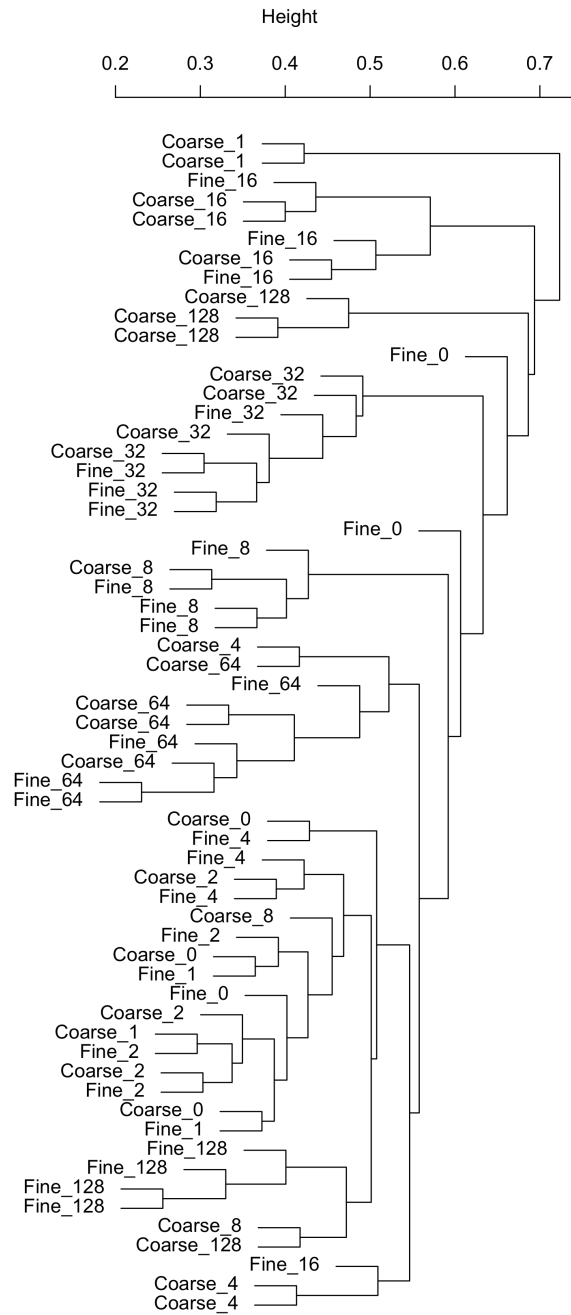
Figure 3.5. Average linkage dendrogram based on Bray-Curtis dissimilarity measures between water oak leaf pack bacterial assemblage RISA profiles of coarse and fine mesh treatments.

CHAPTER IV

INFLUENCE OF LEAF SPECIES ON LITTER BREAKDOWN AND MICROBIAL SUCCESSION IN A SMALL FORESTED STREAM

A. ABSTRACT

Microbial succession during leaf breakdown was investigated using several culture-independent techniques. Red maple and water oak leaves were incubated in a small, forested stream in west-central Georgia, USA, for 128 days, and leaf packs were sampled throughout the incubation period to quantify leaf breakdown rates and microbial community composition. *In situ* breakdown rates were higher for red maple than water oak. Phospholipid fatty acid analysis (PLFA) revealed a significant effect of time on the microbial lipid profiles of both maple and oak. Maple leaf microbial communities contained higher bacterial biomass than fungal biomass, and bacterial biomass increased over the study for both leaf species. Bacterial assemblage structure was examined using complementary molecular methods, including ribosomal intergenic spacer analysis (RISA) and denaturing gradient gel electrophoresis (DGGE). RISA results showed that time in stream was the most important factor structuring bacterial assemblages, with differences between leaf species more distinct at earlier time points. DGGE profiles revealed higher variability in bacterial assemblages of red maple compared to water oak, and sequencing of DGGE-

resolved amplicons indicated the persistent association of *Collimonas* spp. in red maple microbial assemblages, taxa that are frequently known to express chitinase activity. Water oak was dominated over time by *Citrobacter* spp., known for tannic acid degradation. Our results suggest incubation time is the most significant factor influencing leaf litter microbial community composition with differences in leaf species chemistry affecting earlier stages of microbial colonization and these leaf species-specific assemblages dissipating over time.

B. INTRODUCTION

Allochthonous inputs are the major source of energy and nutrients within food webs of many small, forested streams (Webster and Benfield, 1986), with this input primarily entering streams as leaf litter from surrounding riparian vegetation (Abelho, 2001; Fisher and Likens, 1973; Vannote *et al.*, 1980; Webster *et al.*, 1995). Litter breakdown and associated nutrient release mediated by stream microorganisms has been, and continues to be, recognized as a critical process for system metabolism (Abelho, 2001; Minshall, 1967; Young *et al.*, 2008). Allochthonous litter also provides a vital structural habitat for many stream benthic macroinvertebrates (Mackay and Kalff, 1969). Thus, the structural and energetic importance of leaf litter to forested streams makes it an integral part of overall ecosystem integrity and function (Gessner and Chauvet, 2002; Webster and Benfield, 1986; Young *et al.*, 2008).

Leaf breakdown within streams consists of 3 primary phases. First, litter undergoes chemical leaching, which occurs within the first 24 to 48h after

submersion (Petersen and Cummins, 1974). Second, colonization and conditioning by fungi and bacteria makes litter softer and begins to facilitate further decomposition within a few days after submersion (Cummins, 1974; Suberkropp and Klug, 1974). Last, litter is subsequently fragmented by physical abrasion and processing by macroinvertebrate consumers, particularly the shredder functional feeding group (Cummins, 1974; Graca, 2001; Wallace and Webster, 1996), which greatly increase litter breakdown (Wallace *et al.*, 1982).

Leaf chemistry traits, such as initial N concentration, C:N ratio, and lignin, vary among leaf species (Ostrofsky, 1997); such leaf chemistry variation can alter breakdown rates (Petersen and Cummins, 1974). Ostrofsky (1997) showed that the best individual leaf chemistry predictors of breakdown rates were %N, C:N ratio, condensed tannins, and %lignin:%N ratio. Coulson and Butterfield (1978) showed high N, and to a lesser extent P, concentrations were positively correlated to increased microbial densities and breakdown within a bog. High C:N ratios have been shown to be associated with decreased microbial activity and are often found in leaf litter that is high in cellulose and lignin with slower breakdown rates (Witkamp, 1966). In addition, several studies have shown concentrations of structural compounds (e.g., lignin, hemicellulose and cellulose) within litter demonstrate an inverse relationship with litter breakdown, by inhibition of fungal and bacterial colonization of litter (Ardon, 2008; Meentemeyer, 1978; Berg and Staaf, 1980).

Fungal and bacterial species are critical to litter breakdown, and their relative contributions to the leaf conditioning process have been assessed,

indicating a greater contribution by fungi than bacteria with bacterial contribution increasing over time and in the presence of pollution (Hieber and Gessner, 2002; Pascoal and Cássio, 2004). Several studies have characterized fungal and bacterial assemblages present at different stages of leaf breakdown using a combination of cultivation, microscopy, and assaying for reproductive structures and metabolic products (Barlocher and Kendrick, 1974; Suberkropp and Klug, 1976; McArthur *et al.*, 1994; Kreutzweiser and Capell, 2003; Gulis and Suberkropp, 2003).

The advent of molecular techniques, including DNA sequencing and fingerprinting, provides an opportunity to characterize microbial community dynamics during the conditioning process with greater resolution at the microbial identity level, as well as the ability to measure community similarity/dissimilarity (Findlay, 2010). For example, fingerprinting techniques have been used to quantify fungal preferences of leaves during colonization (Nikolcheva *et al.*, 2005), and recent work on bacterial and fungal communities demonstrated the efficacy of denaturing gradient gel electrophoresis (DGGE) in revealing temporal shifts in microbial communities during leaf conditioning (Das *et al.,* 2007; Lyautey *et al.*, 2005; Rees *et al.*, 2006). However, the latter study provided only limited phylogenetic resolution (Das *et al.*, 2007). Phylogenetic resolution of bacterial taxa during the leaf breakdown process would thus contribute greatly to our knowledge of bacterial population dynamics, as well as the role of leaf chemistry as a potential modulator of bacterial assemblage structure during breakdown. Molecular techniques also have known biases, such as differential amplification

of bacterial taxa by varying primer sets, co-migration of ribotypes, or reproducibility of the denaturing gradient. This study sought to reduce the impact of these biases by employing multiple culture-independent techniques.

Our study involved the use of several complementary methods (i.e., DGGE, RISA, PLFA) to quantify dynamics of stream microbial communities during the leaf litter conditioning process. Specifically, sequencing of DGGE ribotype bands was used to increase phylogenetic resolution during litter breakdown by 1) characterizing bacterial succession on leaf litter in a small, forested coastal plains stream, and 2) comparing differences in leaf bacterial communities between leaf species (i.e., water oak and red maple) with strongly contrasting breakdown rates. We hypothesized that fungal lipid abundance would be higher than bacterial lipid abundance for both leaf species, but that water oak would have higher fungal lipid abundance than red maple. In addition, because of differences in leaf chemistry and its influence on breakdown rate, we hypothesized that chemical differences between oak and maple species would be reflected in disparate bacterial assemblages.

C. METHODS

1. Study site

The study was conducted at Kings Mill Creek (UTM 0720701E 3600036N), a second-order, low-gradient stream at the Fort Benning Military Installation (FBMI) in west-central Georgia, USA. FBMI occurs south of the Fall Line in the Sand Hills subecoregion of the Southeastern Plains ecoregion (Griffith

et al., 2001). Kings Mill Creek is a low-nutrient, acidic (pH = 4.33) stream with sandy substrate (Maloney *et al.*, 2005), and an intact deciduous riparian canopy (Houser *et al.*, 2005; Maloney and Feminella, 2006) consisting mostly of red maple (*Acer rubrum*), dogwood (*Cornus* spp.), yellow poplar (*Liriodendron tulipifera*), sweetgum (*Liquidambar styraciflua*), sweetbay magnolia (*Magnolia virginiana*), black gum (*Nyssa sylcatica*), and water oak (*Quercus nigra*) (Cavalcanti, 2004). The Kings Mill Creek watershed was largely forested (85.6% cover, Maloney et al. 2005) with a high abundance of shredder macroinvertebrates (K.O. Maloney, unpubl. data), implying the importance of litter to the stream's trophic economy.

2. Experimental design

An *in situ* litter decomposition experiment was conducted using *Acer rubrum* (red maple) and *Quercus nigra* (water oak) as the two leaf species. These species span a moderate range of breakdown rates, with red maple having a medium breakdown rate (k=0.005-0.010) and water oak a relatively low breakdown rate (k<0.005) (Webster and Benfield, 1986). In addition, maple species show a contrasting leaf chemistry compared to oak species, with maple (*Acer* spp.) having higher N content (low C:N) and oak (*Quercus* spp.) having a higher C:N and lignin content (Ostrofsky, 1997). Both species were common in riparian zones at the study site and at FBMI in general (Lockaby *et al.*, 2005).

We used 9 collection dates (days 0, 1, 2, 4, 8, 16, 32, 64, and 128) from January to May 2007, which included both early microbial colonization and those changes occurring during microbial succession as leaf litter breakdown proceeds.

Leaf packs of both species were placed in mesh bags with mesh size large enough (6.35-mm) to allow macroinvertebrate colonization. Each block consisted of one run habitat containing 4 replicates of each leaf species with each block sampled on 1 of the 9 dates; blocks were chosen randomly during the 128-d study. Artificial leaf packs were held within mesh bags (0.1524 m x 0.3048 m) and placed *in situ*, a common method for studying leaf breakdown in streams (Boulton and Boon, 1991). Leaves were collected from a single tree of each species during fall 2006 (December-January) using tarps strung below trees to accumulate abscissed leaves. Leaves were air-dried in a sterile Class II biosafety cabinet to a constant mass, weighed into 4-g aliquots, and then placed into sterilized mesh bags until deployed. Mesh bags of leaf packs had coarse (6.35 mm) mesh on one side to allow colonization of macroinvertebrates and a smaller (3.175-mm) mesh on the other side to reduce loss of litter particles from inside the bag during incubation. Once filled, mesh bags were sewn closed with nylon and then anchored in the stream with rebar. Leaf species were sampled on day 0 by briefly dipping packs into the stream water and then removing and returning them to the laboratory to quantify handling loss (Petersen and Cummins, 1974). Day 0 packs were selected to represent the initial phyllosphere microbial community for each leaf species, and these packs were treated similarly to all others for further processing.

On the specified collection date, each leaf pack was removed from the block, placed in a Ziploc bag, and returned on ice to the laboratory. A 2-leaf subsample was removed from each leaf pack for microbial processing and the

remaining leaves were used to determine the breakdown rate (below). The leaf subsample was ground in liquid N_2 and stored at –80°C until processed for microbial community characterization (below).

To determine breakdown rate, the remaining leaf litter was rinsed and dried to a constant mass at 60°C, weighed, and then combusted in a muffle furnace at 550°C for 2 h. The ashed residue was weighed and this weight was subtracted from the pre-combusted dry mass for determination of ash-free dry mass (AFDM). Breakdown rates were calculated using an exponential decay model (Petersen and Cummins, 19974) as the slope of the regression line of ln(% AFDM remaining) vs time. Leaf carbon and nitrogen content were measured, covering a range of collection dates including pre-immersion, 1, 16, and 64 days incubation, by thermal combustion using a Perkin-Elmer 2400 CHN Analyzer. Pre-immersion estimates of cellulose and lignin were determined sequentially according to procedures of Van Soest et al. (1991).

To characterize variation in physicochemical conditions known to affect breakdown (Webster and Benfield 1986; Dangles *et al.,* 2004) streamwater temperature, depth, and current velocity were quantified. Temperature was recorded hourly with HOBO Temp data loggers. Differences in depth and current velocity were quantified to assess variation in leaf breakdown and microbial communities attributable to depth or velocity differences. Leaf pack depth was measured using a meter stick placed at the top center of each leaf pack and a Marsh-McBirney Flowmate current meter was used to measure flow within each

leaf pack. Flow inside each leaf pack was measured by positioning an empty "dummy" bag over the probe placed immediately upstream of each leaf pack.

3. Microbial lipids and community characterization

3.1 Microbial lipids. We used phospholipid fatty acid (PLFA) analysis to quantify relative abundance of different lipids associated with bacteria and fungi on litter over the experiment (Zelles, 1999). The PLFA analysis method was adapted from Sasser (1990) for saponification, formation of fatty acid methyl esters (FAMEs), extraction, and a base wash. First, we placed an approximately 680-mg sample of the liquid N_2-ground litter in a 20 mL test tube. Samples were saponified to liberate fatty acids from lipids of lysed cells with 1.0 mL saponification reagent (45 g sodium hydroxide, 150 mL methanol, 150 mL deionized water), vortexed for 10 s, heated to 100°C for 5 min in a boiling water bath, and then vortexed and reheated to 100°C for 25 min. FAMEs were formed through methylation by adding 2 mL of methylating reagent (325 mL 6.0 N HCl, 275 mL methanol), and vortexing and heating them to 80°C for 10 min. FAMEs were extracted from the aqueous phase into an organic phase using 1.25 mL extraction reagent (100 mL hexane, 100 mL methyl-tert butyl ether) tumbled for 10 min. Last, the aqueous phase was removed with a Pasteur pipette, washed with 3 mL of base wash (10.8 g sodium hydroxide, 900 mL distilled water) and tumbled for 5 min. Prior to chromatographic analysis, the organic phase containing FAMEs was transferred to glass vials, and FAMEs were analyzed using the Microbial Identification System (MIDI, Inc., Newark, DE USA). The output provided fatty acid (FA) peak responses for fungi and bacteria from each

sample, which were then used to estimate relative abundance of bacterial and fungal lipids. Based on FA data, we estimated relative fungal and bacterial lipids and used these measures to determine microbial lipid differences between species throughout the study. Relative abundance of bacteria was estimated using branched-chain saturated (e.g. *iso* and *anteiso*), hydroxyl (OH), monounsaturated, and cyclopropyl FAs (Zelles, 1999). Fungal relative abundance was estimated using only three lipid markers (18:2ω6, 18:1ω9c, and 18:3ω6c) (Guckert *et al*., 1985; Vestal and White, 1989).

3.2 DNA extraction for molecular analyses. Leaf subsamples were used for two separate molecular analyses of bacterial communities: 1) ribosomal intergenic spacer analysis (RISA) and 2) DGGE. For these procedures, genomic DNA was extracted from 0.10-g leaf litter using a Qiagen genomic DNA extraction kit (Qiagen, Valencia, CA, USA). DNA was purified using a cetyltrimethylammonium bromide (CTAB) extraction procedure (Ausubel, 1994). In some cases, particularly for leaves at day 0, extracted DNA was not sufficiently pure to serve as template for PCR. For those samples, we conducted an additional round of genomic DNA purification using a combination of 80% formamide and 1M NaCl treatment to provide PCR-ready genomic DNA template (see Chapter II). This formamide purification step has been tested with DNA extracted from many different environments and has not been observed to result in any loss of DNA or corresponding loss of diversity as assessed by DGGE. If this method was deemed necessary by the lack of PCR amplification using DNA

templates derived from commercial kit extraction, then the formamide purification method was consistently applied to all samples from that sampling date.

3.3 RISA analysis of ITS regions. RISA analysis was conducted by PCR amplification of bacterial internal transcribed spacer (ITS) regions and separating polymorphic ITS amplicons within a polyacrylamide gel matrix. PCR was conducted with a reaction volume of 10 μL containing GoGreen Master Mix (Promega; Madison, WI), 1x Bovine Serum Albumin (BSA), nuclease free water, primers, and approx. 1-5 ng genomic DNA template, quantified spectrophotometrically with a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Primers used for these reactions were the universal bacterial primers IRDYE 800-labeled ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') (Cardinale *et al.*, 2004) and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al.*, 2004) at a final concentration of 0.20 μM. This primer set has been shown to not be as susceptible as other primers to known PCR biases such as those due to substrate reannealing (Suzuki and Giovannoni, 1996) and preferential amplification of shorter DNA templates (Cardinale *et al.*, 2004). Amplification was done according to the method of Fisher and Triplett (1999), as follows: reaction mixtures were held at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 15 s, 55°C for 15 s, and 72°C for 45 s and a final extension of 72°C for 2 min. We verified PCR products on a 1% agarose gel stained with ethidium bromide. Following verification of product yield and size, we separated amplicons in a 5.5% polyacrylamide gel matrix and images were recorded using a Li-Cor 4300 (Li-Cor Inc., Lincoln, NE, USA).

3.4 DGGE analysis of 16S rRNA amplicons. For DGGE, replicates from each sampling date were examined using a 2-step process. Genomic DNA extracted from leaves was used as a template in a PCR. Fifty μL reactions were conducted using a GoGreen Master Mix (Promega; Madison, WI) that included Taq polymerase, dNTPs, and Mg^{+2}-containing buffer (at 1x concentration). In addition, the PCR reactions included 5 μL of 1:50 diluted DNA template, 1x BSA and 0.20 μM each of the universal bacterial primer set 518R (5′-ATTACCGCGGCTGCTGG-3′) (Lane, 1991) and 338F-GC (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGA GGCAGCAG-3') (Muyzer *et al.*, 1993). This technique is known to preferentially amplify the most abundant bacterial ribotypes (Muyzer *et al*., 1993), and this specific primer set was chosen to amplify the V3 region of the 16S rDNA, which has been shown to resolve bacterial taxa and produce comparable results to full-length (V1-V9) 16S rDNA gene sequence (Huse *et al*., 2008). PCR conditions included 2-min of denaturation at 95°C followed by 30 cycles of 95°C for 1 min, 1 min of annealing at 55°C, and then 2 min of extension at 72°C (Mullis *et al.*, 1994). An 8% polyacrylamide gel was poured that contained a vertical gradient of formamide and urea at a final gradient concentration range of 45 to 55%. PCR products were loaded in the gel with 20 μL (approx. 198-240 ng) per lane and electrophoresed for 15 h at 100 V and 60°C. After electrophoresis, the gels were stained with ethidium bromide for 10 min and then rinsed in deionized water for 15 min, after which bands were visualized using an AlphaImager HP gel documentation system (Alpha Innotech, San Leandro, CA). Individual bands

were considered distinct ribotypes (Muyzer and Smalla, 1998). Abundant rRNA amplicons for a given sampling time were visually identified, excised, and used as template in a subsequent PCR. All subsequent reactions were in a total volume of 25 µL containing GoGreen Master Mix (Promega; Madison, WI), primers 518R and 338F (without the GC clamp), and 2 µL of excised PCR product. All PCR reactions generated product, without requiring further resolution of bands, and were sequenced using 518R (5 µM) and BigDye sequencing chemistry by the Lucigen Corporation (Middleton, WI). Unaligned sequences were compared to the GenBank nr/nt database using the BLASTn search algorithm at the National Center for Biotechnology Information (NCBI) to obtain the nearest neighbor (≥95% similarity) based on 16S rDNA gene sequence identity. Some studies have found that a portion of 16S rDNA isolated DGGE amplicons, created using universal 16S-rDNA bacterial primers, corresponded to plant 16S rDNA sequences (Kim *et al.*, 2010; Saito *et al.*, 2007). However, no mitochondrial or plastid sequences were obtained from our excised DGGE amplicons.

4. Data analyses

Differences were compared between species and over time in environmental variables and litter breakdown, using 2-way analysis of variance (ANOVA) (Zar, 1999). AFDM data were arcsine transformed prior to analysis to satisfy the assumptions of normality and equality of variance. Current velocity and depth measures were square root transformed prior to analysis to satisfy normality and equality of variance. Overall lipid profiles and bacterial assemblage

composition were compared for each leaf species over time using Analysis of Similarity (ANOSIM) based on a Bray-Curtis dissimilarity matrix. Fungal and bacterial fatty acid abundance estimates were square root transformed and compared for each date using a 2-way ANOVA based on days in stream and leaf species, as well as Tukey's multiple comparisons within each time point. RISA gel images were analyzed using BioNumerics Software v5.0 (Applied Maths, Kortrijk, Belgium) to quantify bacterial assemblage similarity. Bands were defined relative to the highest band density on that pattern, where all bands with a density >10% of the highest band density were used to create a presence-absence matrix for further analysis. Similarities between band presence-absence fingerprints were calculated using Jaccard's similarity coefficient. Cluster analysis using Ward's method was then used to create dendrograms for visualization of bacterial assemblage similarity (Saitou and Nei, 1987). A variety of clustering algorithms were compared, and Ward's method of hierarchical clustering yielded the most satisfactory result. An alpha level of 0.05 was used for all statistical analyses.

D. RESULTS

1. Physicochemical conditions

Water temperature during the 128-d study (January to May) ranged from 3.7 to 30.3^{o}C, with a mean temperature of 13.4^{o}C over the entire incubation period. Mean water depth at individual leaf packs was 0.17 m, which did not differ between species (F=0.43, p=0.515). Mean current velocity immediately upstream

of leaf packs was 0.07 m/s, which also did not differ between species (F=3.73, p=0.059). Mean water depth at leaf packs decreased significantly (p=<0.001) from 0.31 m on day 1 to 0.09 m on day 128. Mean current velocity also varied significantly over the study (p=<0.001) with the highest velocity experienced on day 4 (0.11 m/s) and the lowest on day 16 (0.02 m/s). The degree to which pH and oxygen varied over the course of this study was not measured. However, streamwater pH and dissolved oxygen measurements on day 1 indicated the stream was acidic (pH = 4.33), but well oxygenated (8.65 mg/L, 88% saturation).

2. Litter breakdown

Mean leaf litter breakdown rates for red maple (hereafter maple) and water oak (hereafter oak) over the study were k = 0.075 d^{-1} for maple and k = 0.026 d^{-1} for oak (Fig. 4.1). Maple breakdown was significantly faster than oak (p<0.001). The exponential decay model explained 83.6 and 63.1% of the variation in maple and oak leaf breakdown, respectively (Fig. 4.1). Maple AFDM decreased rapidly from 100% AFDM remaining at day 0 to 81.22% after 1 day in situ; in contrast, oak showed little AFDM change during the same 1-d interval (~2% loss). After 128 d, maple had 46.6% AFDM remaining, compared to 73.9% remaining for oak.

Oak leaves also had a higher nitrogen content than maple (1.276% vs. 0.43%) and a lower C:N ratio (35.43) than maple (106.83). Nitrogen content of immersed oak leaves decreased over the study from 0.52% following 1 day instream incubation to 0.44% after 64 days; whereas, maple leaf nitrogen content increased from 0.20% on day 1 to 0.32% on day 64. Non-immersed oak leaves

contained higher cellulose (21.31%) and lignin (14.63%) compared to maple

leaves (10.87% and 6.68%, respectively).

3. Microbial community characterization

Overall, lipid profiles varied significantly over time for both maple

(p=0.001) and oak (p=0.005). Bacterial and fungal lipid relative abundance,

estimated by FAME analysis, significantly differed between maple and oak

on all dates except day 128 (Fig. 4.2 and Fig. 4.3). Overall, bacterial lipid

abundance on maple was higher than oak (p<0.001) (Fig. 4.2), whereas oak

showed higher abundance of fungal lipids than maple (p<0.001) (Fig. 4.3).

Fungal lipid relative abundance on oak leaves tended to decrease over the

128-d incubation, as bacterial lipids steadily increased (Fig. 4.3).

Analysis of bacterial assemblages in leaf packs using RISA

demonstrated a dependence of community structure upon time of incubation

(p<0.001) (Fig. 4.4). The degree to which leaf species played a role in

structuring bacterial assemblages, although significant overall (p=0.03),

tended to vary with time. Cluster analysis indicated assemblage structure

apportioned into 3 temporal groupings, pre-immersion, early breakdown,

and later breakdown assemblages (Fig. 4.4). However, within each

grouping, leaf species appeared to play a greater role structuring bacterial

assemblages during pre-immersion (day 0) (p=0.004) than during later

breakdown (day 128) (p=0.103).

Overall bacterial ribotype evenness, as revealed by DGGE, was

higher for maple than oak, with 21 distinct ribotypes on maple (Fig. 4.5) and

18 on oak (Fig. 4.6). The highest ribotype evenness for maple (14) was on day 32, whereas evenness on oak was highest on days 0 and 1 (10 and 16 ribotypes, respectively). Overall, seven different bacterial ribotypes were considered abundant or consistent members of maple leaf packs and were selected for excision and sequencing. Bacterial members of maple litter included the genera *Ralstonia* (*β-Proteobacteria*; day 0), *Sphingopyxis* (*α-Proteobacteria*; days 1 and 32), *Comamonas* (*β-Proteobacteria*; day 4), *Herbaspirillum* (*β-Proteobacteria*; day 4), *Mesorhizobium* (*α-Proteobacteria*; day 4), *Nitrosospira* (*β-Proteobacteria*; day 8), and *Collimonas* spp. (*β-Proteobacteria*; days 16, 64, and 128) with percent sequence identities to GenBank matches ranging from 95 to 100%. In contrast, the oak litter bacterial assemblage was less variable than maple, as indicated by less ribotype variation over the study. The genus *Citrobacter* (*γ-Proteobacteria*) occurred on oak leaves on all dates. Genera from five other ribotypes also were dominant including *Mesorhizobium* (*α-Proteobacteria*; day 0), *Sphingomonas* (*α-Proteobacteria*; day 1), *Aquabacterium* (*β-Proteobacteria*; day 8), *Sphingopyxis* (*α-Proteobacteria*; days 0), and *Thiobacillus* (*β-Proteobacteria*; day 128) with percent sequence identities to GenBank matches ranging from 95 to 100%.

E. DISCUSSION

Leaf breakdown rates strongly differed between species (i.e., fast for maple and slower for oak), a result that was consistent with previous work (Webster and Benfield, 1986). Such differences reflect contrasting leaf chemistry,

with higher lignin content likely contributing to slower breakdown and resulting in differences in microbial communities between these leaf species (Webster and Benfield, 1986; Gessner and Chauvet, 1994; Ostrofsky, 1997). Similar to previous studies (Das *et al.*, 2007), lipid profiles of both leaf species varied significantly with incubation time. Maple leaf packs showed higher abundance of bacterial lipids compared to oak. This difference could be a function of oak having lower surface area available for colonization by bacteria than maple as well as having higher lignin content making it harder for bacteria to colonize (Das *et al.*, 2007). In addition, the faster breakdown of maple could increase nutrient availability on leaf surfaces and thus stimulate bacterial growth. Alternatively, oak showed higher fungal marker abundance than maple on most sampling dates, possibly because fungi are more capable of colonizing lignin-rich oak leaves than bacteria and tend to dominate for a longer period; in contrast, on maple leaves, with less lignin, bacteria are capable of earlier colonization following fungal conditioning and potential fungal degradation, thus potentially reducing fungal biomass (Gessner and Chauvet, 1994; Gulis and Suberkropp, 2003). Fungal lipid marker abundance on oak did decrease over the 128-d incubation (Fig. 3.3), so increased bacterial lipid abundance on oak leaves may have resulted from increased nutrient availability for bacteria following fungal colonization and conditioning (Gulis and Suberkropp, 2003).

RISA results indicated that bacterial assemblage structure was more influenced by incubation time than by leaf species. These results, as well as our microbial lipid profiles, agree with a study by Das *et al*. (2007) that found that

time was a key factor in structuring fungal, bacterial, and actinomycete assemblages compared to the influence of leaf species. The greater impact of time on assemblage structure could be, in part, due to observed temporal variation in streamwater environmental conditions (e.g., temperature, water depth, and current velocity). Slight variations in streamwater conditions could favor some bacterial taxa over others potentially altering bacterial assemblage structure as breakdown proceeded. The lower degree of separation between maple and oak bacterial assemblages during later breakdown (days 128) likely is attributable to plant compounds (e.g. tannins, phenolics, etc.) present in higher quantities earlier in the breakdown process that leached out and/or diminished in quantity. Canhoto and Graca (1999) demonstrated the inhibitory effects of secondary compounds, such as tannic acid, on microbial biomass. In their study, decreased growth of four different aquatic hyphomycetes was observed following the addition of increasing concentrations of tannic acid and eucalyptus oils. In our study, chemical differences between maple and oak would likely be at their highest during the initial days of incubation prior to significant leaching; thus, our observations of the most extreme separation between maple and oak bacterial assemblages occurring during these earlier stages of breakdown are consistent with this mechanism. This increased similarity between maple and oak bacterial assemblages over time could also be a reflection of increased bacterial colonization on oak leaves following fungal conditioning, permitting colonization of leaf litter-associated bacteria that were previously incapable of colonizing the oak leaf surface due to increases in exposed surface area. The ability of fungal

activity to increase bacterial colonization has been suggested (Suberkropp and Klug, 1976), and prior studies have also shown increased bacterial colonization in response to increased surface area and available organic matter (Yamamoto and Lopez, 1985).

DGGE results of dominant taxa revealed a more diverse and variable bacterial assemblage associated with maple compared to oak. Increasing similarity over time between maple and oak bacterial assemblages, as indicated by the RISA results, indicates the presence of similar bacterial taxa on red maple and water oak leaf litter. However, when comparing the abundant bacterial taxa identified via DGGE, a trend towards increasing *Collimonas* spp. was observed with maple assemblages, whereas a more consistent high relative abundance of *Citrobacter* spp. was observed in oak assemblages. Although these two specific taxa were different between oak and maple leaves, other abundant and sequenced taxa were shared between leaf species (e.g. *Mesorhizobium*, *Sphingopyxis*). Das *et al*. (2007) also found a band (ribotype) that was only found on sugar maple leaves and one that was only found on white oak leaves. Although each leaf species does seem to have its own leaf species-specific taxa, increased similarity over time between leaf species is likely due to increased colonization by bacterial taxa of lower abundance.

Abundance of bacterial taxa within the genus *Collimonas* associated with maple leaf litter suggests the presence of chitinolytic bacteria, as many *Collimonas* spp. are known to express chitinase activity (Fritsche *et al*., 2008). *Collimonas* spp. presence occurred concurrently with a decrease in fungal lipid

marker abundance, potentially resulting from increased fungal chitin degradation (Fig. 4.5, ribotype G). In contrast, a less variable bacterial assemblage associated with oak had a high relative abundance of *Citrobacter* spp. Many species within the genus *Citrobacter*, such as *Citrobacter freundii*, have the ability to degrade tannic acid (Fig. 4.6, ribotype J) (Murugan *et al.*, 2008). This is significant given that oak (*Quercus* spp.) leaves tend to have relatively high concentrations of hydrolysable tannins (Zimmer *et al.*, 2002), which in turn form complexes with other macromolecules to create tannic acid. In addition, oak leaves have been shown to have slow leaching rates of phenolics, likely due to their thick cuticle (Kuiters and Sarink, 1986), and would potentially explain why *Citrobacter* spp. were found in association with oak leaves on all sampling dates. It is also important to note that PCR amplification may under represent ribotype richness (Acinas *et al.*, 2005), so bacterial taxa observed in this analysis of maple and oak leaves are likely only the more abundant ribotypes at their respective dates of incubation; therefore, it is expected that a much greater bacterial taxa richness at lower relative abundance exists within these bacterial assemblages.

Our results showed a more variable bacterial assemblage in fast breakdown leaf species (i.e., red maple), whereas slower degrading species such as water oak were dominated by higher fungal lipid marker abundance and a less variable bacterial assemblage. However, as leaf litter leachate rates decrease over time, colonization by additional bacterial species on oak may become less constrained by leaf chemistry differences and become more

phylogenetically diverse, exhibiting properties more similar to the bacterial assemblages of fast species such as red maple, whose leachate is more easily dispersed and are more readily colonized. Overall, our results suggest leaf chemistry differences play a role early in the breakdown process prior to leaching and colonization by lesser abundant bacterial taxa, yet the main factor controlling microbial community structure appears to be incubation time.

Future studies should examine microbial succession within leaf microbial communities in relation to changes in various environmental factors, particularly examining the effect of anthropogenic disturbances. Disturbance frequently leads to alteration of instream environmental conditions and leaf breakdown, so it is likely that such alterations also affect associated microbial communities given the extreme sensitivity of microbes to environmental change (Young *et al.,* 2008; Ager *et al.*, 2010). Enhanced understanding of the structural dynamics and functional roles of microorganisms in streams also will enable a better understanding of the impacts of disparate environmental influences on ecosystem-level processes.

F. LITERATURE CITED

Abelho, M. 2001. From litterfall to breakdown in streams: a review. *Scientific World Journal*, 1, 656-680.

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V. and Polz, M.F. 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Applied Environmental Microbiology*, 71, 8966-8969.

Ager, D., Evans, S., Li, H., Lilley, A.K. and van der Gast, C. 2010. Anthropogenic disturbance affects the structure of bacterial communities. *Environmental Microbiology*, 12, 670-678.

Ausubel, F.M. 1994. Preparation of plant DNA using CTAB. *Current Protocols in Molecular Biology,* Suppl 27, 2.3-2.3.7.

Bärlocher, F., Seena, S., Wilson, K.P. and Williams, D.D. 2008. Raised water temperature lowers diversity of hyporheic aquatic hyphomycetes. *Freshwater Biology*, 53, 368-379.

Berg, B.R. 2000. Litter decomposition and organic matter turnover in northern forest soils. *Forest Ecology and Management*, 133, 13-22.

Berg, B.R. and Tamm, C.O. 1991. Decomposition and nutrient dynamics of litter in long-term optimum nutrition experiments. *Scandinavian Journal of Forest Research*, 6, 305-321.

Boulton, A.J. and Boon, P.I. 1991. A review of methodology used to measure leaf litter decomposition in lotic environments: time to turn over an old leaf? *Marine and Freshwater Research*, 42, 1-43.

Canhoto, C. and Graça, M.A.S. 1999. Leaf barriers to fungal colonization and

    shredders *Tipula lateralis*. consumption of decomposing *Eucalyptus*

    globules. *Microbial Ecology*, 37, 163-172.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A.,

    Zanardini, E., Sorlini, C., Corselli, C. and Daffonchio, D. 2004.

    Comparison of different primer sets for use in automated ribosomal

    intergenic spacer analysis of complex bacterial communities. *Applied and*

    Environmental Microbiology, 70, 6147-6156.

Cavalcanti, G. 2004. Effects of sediment deposition on aboveground net primary

    productivity, vegetation composition, structure, and fine root dynamics in

    riparian forests. M.S. Thesis, Auburn University, Auburn, AL.

Dang, H. and Lovell, C.R. 2000. Bacterial primary colonization and early

    succession on surfaces in marine waters as determined by amplified rRNA

    gene restriction analysis and sequence analysis of 16S rRNA genes.

    Applied and Environmental Microbiology, 66, 467-475.

Dangles, O., Gessner, M.O., Guerold, F. and Chauvet, E. 2004. Impacts of

    stream acidification on litter breakdown: implications for assessing

    ecosystem functioning. *Ecology*, 41, 365-378.

Das, M., Royer, T.V. and Leff, L.G. 2007. Diversity of fungi, bacteria, and

    actinomycetes on leaves decomposing in a stream. *Applied and*

    Environmental Microbiology, 73, 756-767.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K.,

    Huber, T., Dalevi, D., Hu, P., and Andersen, G.L. 2006a. Greengenes, a

Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Applied and Environmental Microbiology* 72, 5069-72.

DeSantis, T. Z., Hugenholtz, P., Keller, K., Brodie, E.L., Larsen, N., Piceno, Y.M., Phan, R., and Andersen, G.L. 2006b. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res* 34, W394-399.

Fisher, S.G. and Likens, G.E. 1973. Energy flow in Bear Brook, New Hampshire: an integrative approach to stream ecosystem metabolism. *Ecological monographs,* 43, 421-439.

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

Fritsche, K., De Boer, W., Gerards, S., Van Den Berg, M., Van Veen, J.A. and Leveau, J.H.J. 2008. Identification and characterization of genes underlying chitinolysis in *Collimonas fungivorans* Ter331. *FEMS Microbiology Ecology*, 66, 123-135.

Gartner, T.B. and Cardon, Z.G. 2004. Decomposition dynamics in mixed-species leaf litter. *Oikos*, 104. 230-246.

Gessner, M.O. and Chauvet, E. 1994. Importance of stream microfungi in controlling breakdown rates of leaf litter. *Ecology*, 75, 1807-1817.

Gessner, M.O. and Chauvet, E. 2002. A case for using litter breakdown to assess functional stream integrity. *Ecological Applications*, 12, 498-510.

Gessner, M.O., Chauvet, E. and Dobson, M. 1999. A perspective on leaf litter breakdown in streams. *Oikos*, 85, 377.

Gleave, A. P., R. K. Taylor, B. A. M. Morris, and D. R. Greenwood. 1995. Cloning and sequencing of a gene encoding the 69-kDa extracellular chitinase of *Janthinobacterium lividum*. FEMS Microbiol. Lett. 131, 279–288.

Graca, M.A.S. 2001. II. Leaf litter processing and invertebrates: The role of invertebrates on leaf litter decomposition in streams – a review. *International Review of Hydrobiology*, 86, 383-393.

Greathouse, E.A., March, J.G. and Pringle, C.M. 2005. Recovery of a tropical stream after a harvest-related chlorine poisoning event. *Freshwater Biology*, 50, 603-615.

Griffith, G.E., Omernik, J.M., Comstock, J.A., Lawrence, S., Martin, G., Goddard, A., Hulcher, V.J., and Foster, T. 2001. Ecoregions of Alabama and Georgia, color poster with map, descriptive text, summary tables, and photographs: Reston, Virginia, U.S. Geological Survey map scale 1:1,700,000.

Gulis, V. and Suberkropp, K. 2003. Interactions between stream fungi and bacteria associated with decomposing leaf litter at different levels of nutrient availability. *Aquatic Microbial Ecology*, 30, 149-157.

Hättenschwiler, S., Aeschlimann, B., Coûteaux, M.-M., Roy, J. and Bonal, D. 2008. High variation in foliage and leaf litter chemistry among 45 tree species of a neotropical rainforest community. *New Phytologist*, 179, 165-175.

Hieber, M. and Gessner, M.O. 2002. Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. *Ecology*, 83, 1026-1038.

Hoisington, D. Khairallah, M. and Gonzalez-de-Leon, D. 1994. Laboratory Protocols: CIMMYT Applied Biotechnology Center. Second Edition, Mexico, D.F.: CIMMYT.

Houser, J.N., Mulholland, P.J. and Maloney, K.O. 2005. Catchment disturbance and stream metabolism: patterns in ecosystem respiration and gross primary production along a gradient of upland soil and vegetation disturbance. *Journal of the North American Benthological Society*, 24, 538-552.

Huse, S.M., Dethlefsen, L., Huber, J.A., Mark Welch, D., Relman, D.A. and Sogin, M.L. 2008. Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *PLoS Genetics*, 4(11): e1000255.

Jenkins, C.C. and Suberkropp, K. 1995. The influence of water chemistry on the enzymatic degradation of leaves in streams. *Freshwater Biology*, 33, 245-253.

Kabagale, A.C., Cornu, B., van Vliet, F., Meyer, C-L., Mergeay, M., Simbi, J-B.L., Droogmans, L., Wauven, C.V. and Verbruggen, N. 2010. Diversity of endophytic bacteria from the cuprophytes *Haumaniastrum katangense* and *Crepidorphopalon tenuis*. *Plant Soil*, 334, 461-474.

Kim, S-Y., Yoo, K-S., Kim, J.E., Kim, J-S., Jung, J.Y., Jin, Q., Eom, H-J., and Han, N.S. 2010. Diversity analysis of lactic acid bacteria in Korean rice

wines by culture-independent method using PCR-denaturing gradient gel

electrophoresis. *Food Science and Biotechnology*, 19, 749-755.

Kinkel, L.L. 1997. Microbial population dynamics on leaves. *Annual Review of Phytopathology*, 35, 327-347.

Klappenbach, J.A., Saxman, P.R., Cole, J.R. and Schmidt, T.M. 2001. rrnDB: the Ribosomal RNA Operon Copy Number Database. *Nucleic Acids Research*, 29, 181-184.

Kreutzweiser, D.P. and Capell, S.S. 2003. Benthic microbial utilization of differential dissolved organic matter sources in a forest headwater stream. *Canadian Journal of Forest Research*, 33, 1444-1451.

Kuiters, A.T. and Sarink, H.M. 1986. Leaching of phenolic compounds from leaf and needle litter of several deciduous and coniferous trees. *Soil Biology and Biochemistry,* 18, 475-480.

Lane, D.J. 1991. 16S/23S rRNA sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics*. Eds. E. Stackebrandy and M. Goodfellow, pp. 115-175. John Wiley and Sons, New York.

Lockaby, B.G., Governo, R., Schilling, E., Cavalcanti, G. and Hartsfield, C. 2005. Effects of sedimentation on soil nutrient dynamics in riparian forests. *Journal of Environmental Quality*, 34, 390-396.

Lyautey, E., Jackson, C.R., Cayrou, J., Rols, J.L. and Garabetian, F. 2005. Bacterial community succession in natural river biofilm assemblages. *Microbial Ecology*, 50, 589-601.

Mackay, R.J. and Kalff, J. 1969. Seasonal variation in standing crop and species

diversity of insect communities in a small Quebec stream. *Ecology*, 50, 101-109.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Jr., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M. and Tiedje, J.M. 2001. The RDP-II Ribosomal Database Project. *Nucleic Acid Research*, 29, 173-174.

Maloney, K.O. and Feminella, J.W. 2006. Evaluation of single- and multi-metric benthic macroinvertebrate indicators of catchment disturbance over time at the Fort Benning Military Installation, Georgia, USA. *Ecological Indicators*, 6, 469-484.

Maloney, K.O., Mulholland, P.J. and Feminella, J.W. 2005. Influence of catchment-scale military land use on stream physical and organic matter variables in small Southeastern Plains catchments USA. *Environmental Management*, 35, 677-691.

McArthur, J.V., Aho, J.M., Rader, R.B. and Mills, G.L. 1994. Interspecific leaf interaction during decomposition in aquatic and floodplain ecosystems. *Journal of the North American Benthological Society*, 13, 57-67.

Mullis, K.B., Ferrè, F., Gibbs, R.A. and Watson, J.D. 1994. *The polymerase chain reaction*. Birkhauser, Boston.

Murugan, S., Devi, P.U., Mahesh, P., Suja, S. and Mani, K.R. 2008. Production of tannase by Citrobacter freundii under solid state fermentation and its application in fruit juices debittering. *Biosciences Biotechnology Research Asia*, 5, 301-306.

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

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

Nikolcheva, L.G., Bourque, T. and Bärlocher, F. 2005. Fungal diversity during initial stages of leaf decomposition in a stream. *Mycological Research*, 109, 246-253.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M. and Liesack, W. 2005. Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environmental Microbiology*, 7, 382-395.

Ostrofsky, M.L. 1997. Relationship between chemical characteristics of autumn-shed leaves and aquatic processing rates. *Journal of the North American Benthological Society*, 16, 750-759.

Petersen, R.C. and Cummins, K.W. 1974. Leaf processing in a woodland stream. *Freshwater Biology*, 4, 343-368.

Preston, C.M., Trofymow, J.A., Niu, J. and Fyfe, C.A. 2002. Harvesting and climate effects on organic matter characteristics in British Columbia coastal forests. *Journal of Environmental Quality*, 31, 402-413.

Rees, G.N., Watson, G.O., Baldwin, D.S. and Mitchell, A.M. 2006. Variability in sediment microbial communities in a semipermanent stream: impact of

drought. *Journal of the North American Benthological Society*, 25, 370-378.

Saito, A., Ikeda, S., Ezura, H. and Minamisawa, K. 2007. Microbial community analysis of the phytosphere using culture-independent methodologies. *Microbes and Environments*, 22, 93-105.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406-425.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. In: *Technical Note #101.* Microbial ID, Newark, DE.

Schloss, P.D. and Handelsman, J. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology*, 71, 1501-1506.

Shrestha, P.M., Noll, M. and Liesack, W. 2007. Phylogenetic identity, growth-response time and rRNA operon copy number of soil bacteria indicate different stages of community succession. *Environmental Microbiology*, 9, 2464-2474.

Suberkropp, K. and Klug, M.J. 1976. Fungi and bacteria associated with leaves during processing in a woodland stream. *Ecology*, 57, 707-719.

Trofymow, J.A., Moore, T.R., Titus, B., Prescott, C., Morrison, I., Siltanen, M., Smith, S., Fyles, J., Wein, R. and Camirè, C. 2002. Rates of litter decomposition over 6 years in Canadian forests: influence of litter quality and climate. *Canadian Journal of Forest Research*, 32, 789-804.

Wallace, J.B., Webster, J.R. and Cuffney, T.F. 1982. Stream detritus dynamics: regulation by invertebrate consumers. *Oecologia*, 53, 197-200.

Wallace, J.B., Eggert, S.L., Meyer, J.L. and Webster, J.R. 1997. Multiple trophic levels of a forest stream linked to terrestrial litter inputs. *Science*, 277, 102-104.

Webster, J.R. and Benfield, E.F. 1986. Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics*, 17, 567-594.

Wright, M.S. and Covich, A.P. 2005. The effect of macroinvertebrate exclusion on leaf breakdown rates in a tropical headwater stream. *Biotropica*, 37, 403-408.

Yamamoto, N. and Lopez, G. 1985. Bacterial abundance in relation to surface area and organic content of marine sediments. *Journal of Experimental Marine Biology and Ecology,* 90, 209-220.

Young, I.M., Crawford, J.W., Nunan, N., Otten, W., Spiers, A. and Donald, L.S. 2008. Chapter 4 Microbial Distribution in Soils: Physics and Scaling. In: *Advances in Agronomy*. pp. 81-121. Academic Press.

Zar, J.H. 1999. *Biostatistical analysis*. 4th ed. Prentice-Hall, Upper Saddle River, NJ.

Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biology and Fertility of Soils*, 29, 111-129.

Zimmer, M., Pennings, S.C., Buck, T.L. and Carefoot, T.H. 2002. Species specific patterns of litter processing by terrestrial isopods (Isopoda:

Oniscidea) in high intertidal salt marshes and coastal forests. *Functional Ecology,* 16, 596-607.
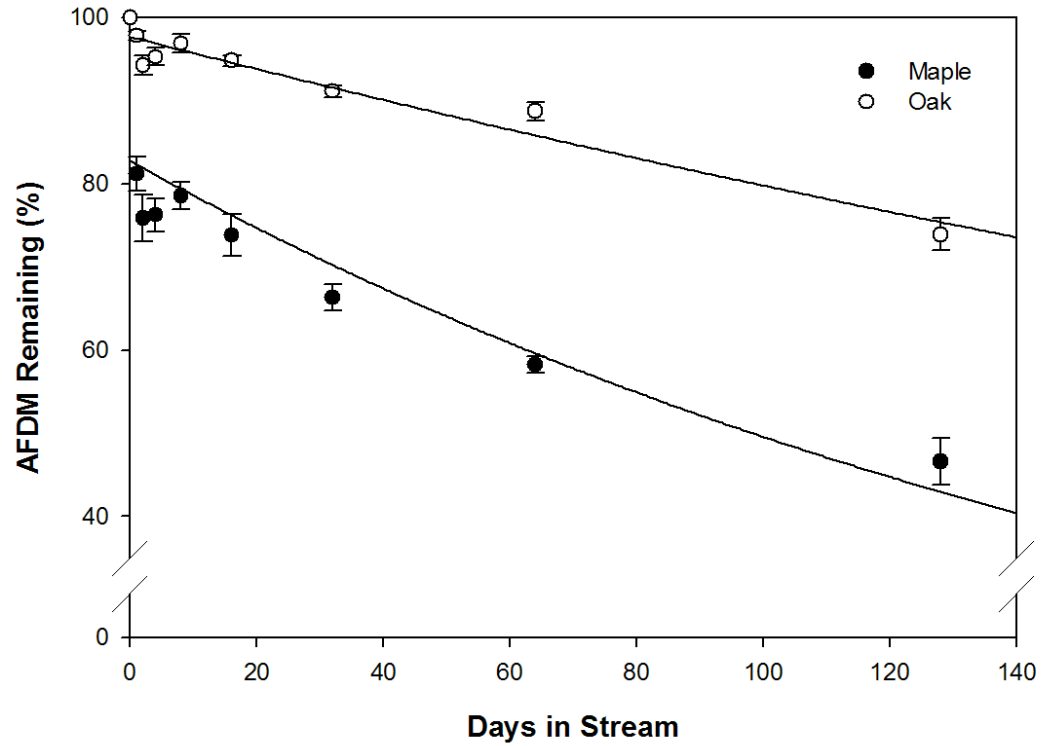
Figure 4.1. Ash free dry mass (AFDM) remaining over time during breakdown of red maple (o) and water oak (•) leaf packs incubated for 128 days in Kings Mill Creek, GA, USA. Plotted points are means (± 1SE).
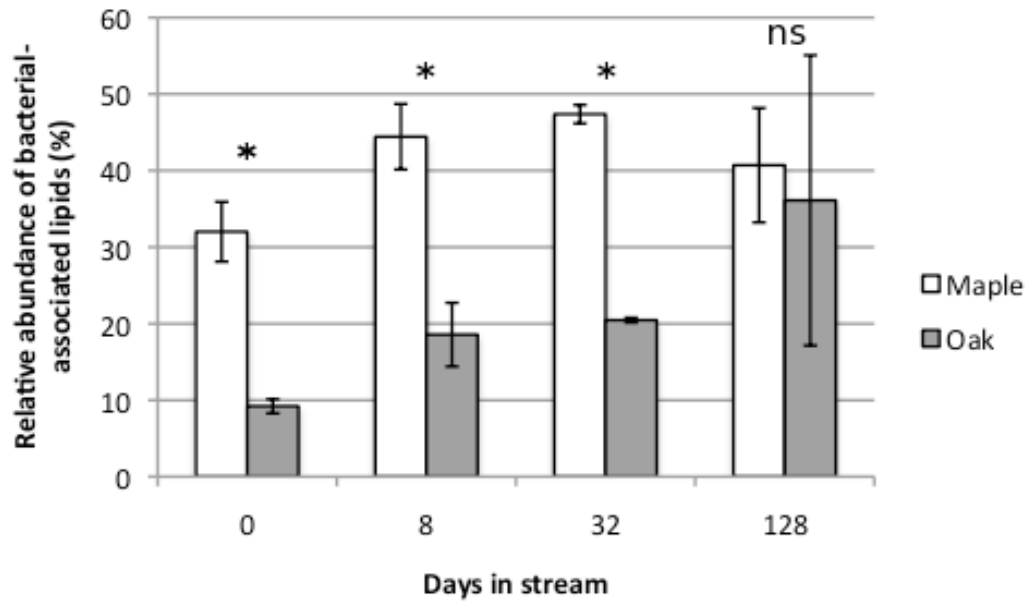
Figure 4.2. Relative abundance (%) of bacterial lipid markers of red maple and water oak leaf packs over the 128-d incubation in Kings Mill Creek, GA, USA. Points on graph represent mean relative abundance (%) ± 1SE. (* = $p<0.001$, ns = not significantly different)
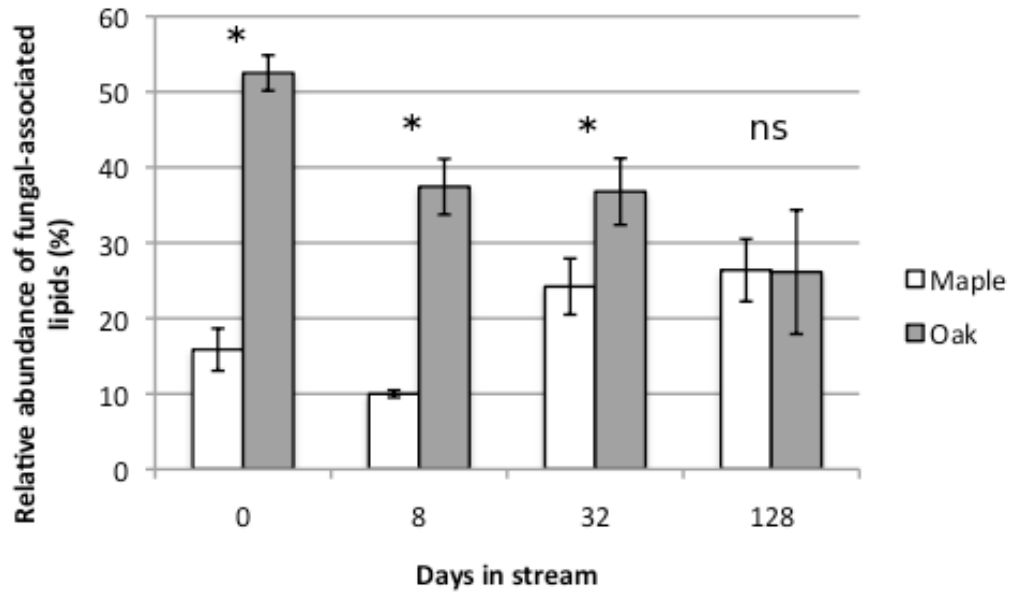
Figure 4.3. Relative abundance (%) of fungal lipid markers of red maple and water oak leaf packs over the 128-d incubation In Kings Mill Creek, GA, USA. Points on graph represent mean relative abundance (%) ± 1SE. (* = $p<0.001$, ns = not significantly different)
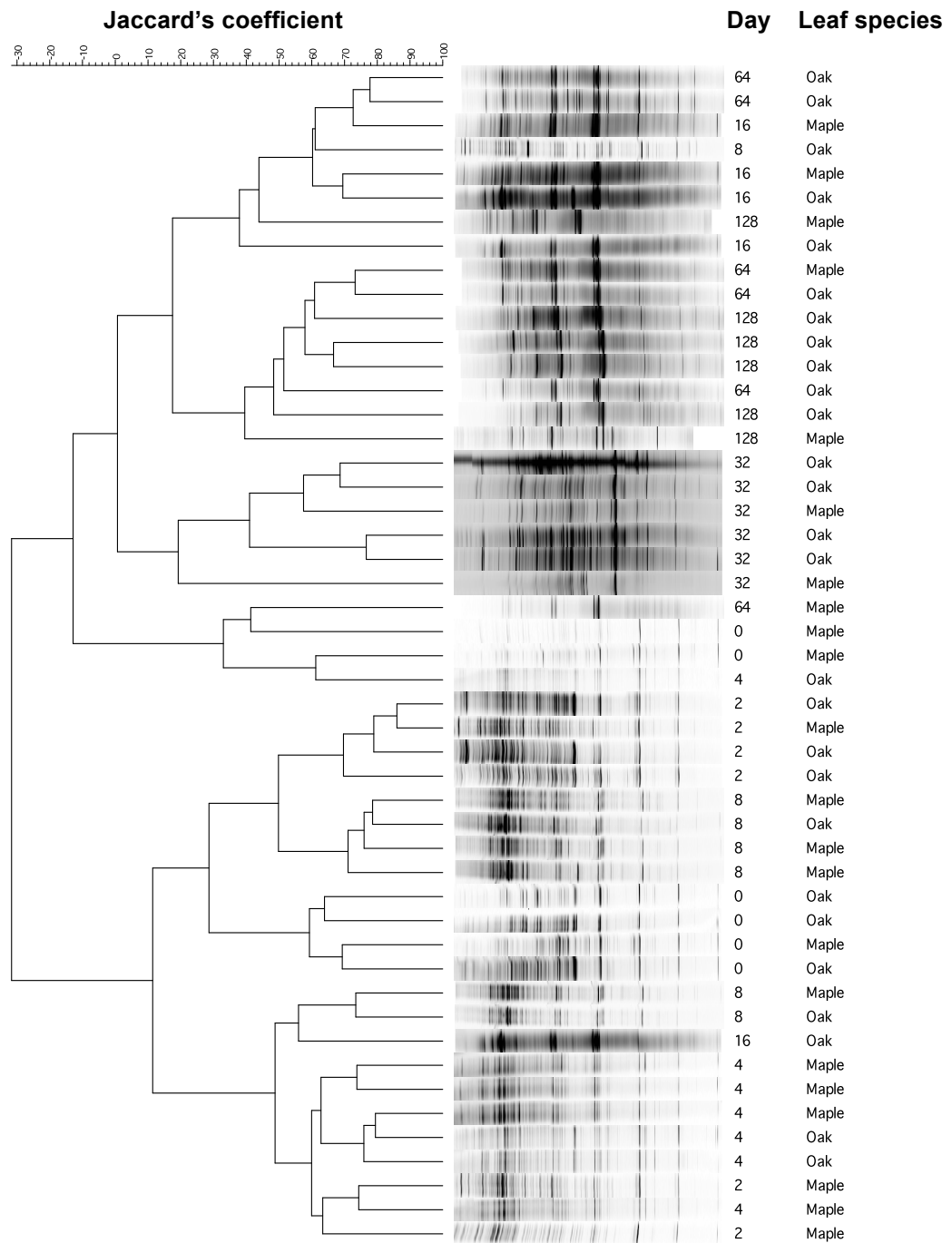
Figure 4.4. Dendrogram of RISA electropherograms displaying bacterial assemblage similarities calculated using Ward's method based on Jaccard's similarity coefficient.

RED
MAPLE

0   1   1   4   4   8   8   0   16   16   32   32   64   128   0

A
B
C
D
E

F
B

G
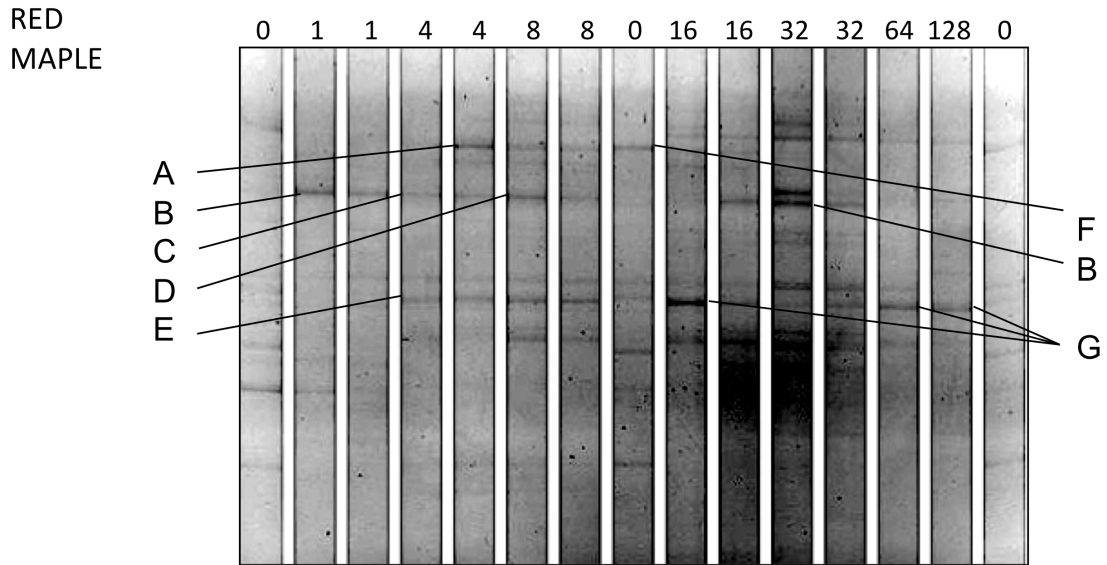
Figure 4.5. Denaturing gradient gel electrophoresis (DGGE) analysis of red maple leaf pack bacterial assemblages. Upper case letters indicate sequenced ribotypes. (Ribotype key: A = *Comamonas*, B = *Sphingopyxis*, C = *Herbaspirillum*, D = *Nitrosospira*, E = *Mesorhizobium*, F = *Ralstonia*, G = *Collimonas*)
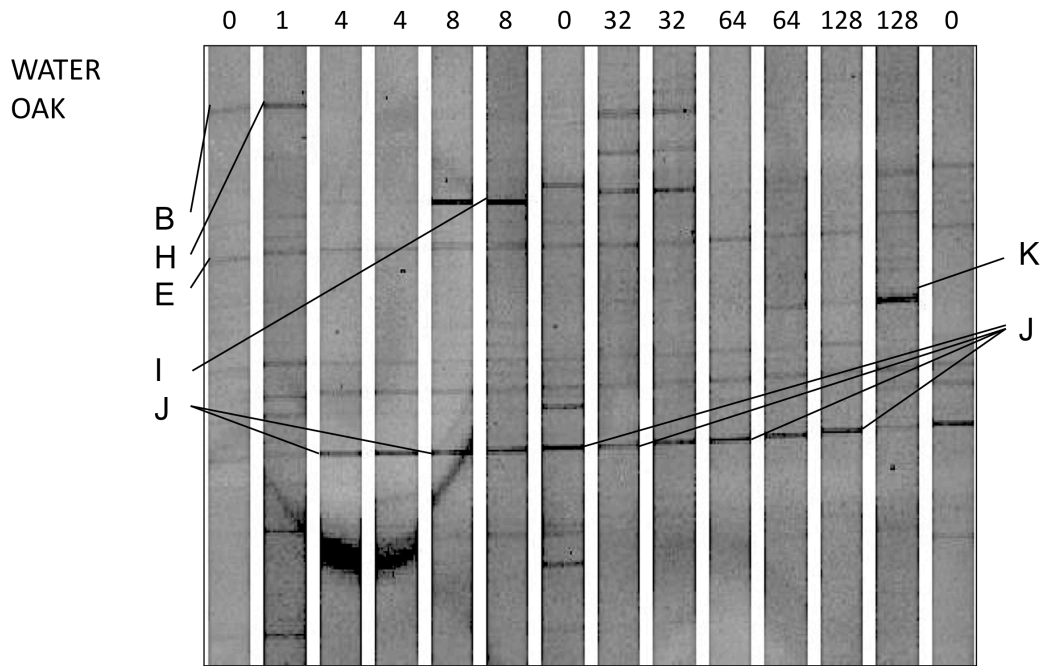
Figure 4.6. Denaturing gradient gel electrophoresis (DGGE) analysis of water oak leaf pack bacterial assemblages. Upper case letters indicate sequenced ribotypes. (Ribotype key: B = *Sphingopyxis*, E = *Mesorhizobium*, H = *Sphingomonas*, I = *Aquabacterium*, J = *Citrobacter*, K = *Thiobacillus)*

CHAPTER V

EFFECTS OF SEDIMENT DISTURBANCE ON BACTERIAL ASSEMBLAGE ASSOCIATED WITH LEAF BREAKDOWN IN SMALL COASTAL PLAINS STREAMS

A. ABSTRACT

This study investigated the effects of sediment disturbance via upland land use on the bacterial assemblage of decomposing leaf litter. A 64-day *in situ* breakdown study was conducted using red maple and water oak leaf packs incubated within six streams (3 low- and 3 high-sediment disturbance) in west-central Georgia, USA. Leaf packs were sampled throughout the incubation and used to calculate breakdown rates as well as to characterize the leaf litter-associated macroinvertebrate and bacterial assemblages. *In situ* breakdown rates of maple were slower under high-sediment disturbance. High-sediment disturbance leaf packs of both leaf species had significantly less macroinvertebrate abundance, density, taxa richness, Shannon's diversity, and biomass. The bacterial assemblage structure of red maple leaf packs was examined using both ribosomal intergenic spacer analysis (RISA) and 454 bar-coded pyrosequencing. Both RISA and pyrosequencing results showed significant separation between low- and high-sediment disturbance leaf pack

bacterial assemblage composition following 32 and 64 days instream incubation. Pyrosequencing results revealed an increased relative abundance of taxa within the phylum *Acidobacteria* in low-sediment disturbance leaf packs. High-sediment disturbance leaf packs had an increased abundance of taxa within the phyla *Firmicutes* (Clostridiaceae) and *delta-Proteobacteria* (Geobacteraceae). Both low- and high-sediment disturbance leaf packs decreased in the relative abundance of *gamma-Proteobacteria* (Pseudomonadaceae) between 32 and 64 days. Thirty-one percent of the variation in bacterial assemblage composition was significantly explained by streamwater pH, which also correlated with disturbance intensity. Our results suggest a shift in leaf pack bacterial assemblage composition under increased sedimentation and catchment disturbance intensity toward a bacterial assemblage dominated by taxa capable of withstanding harsh environmental conditions (e.g. increased streamwater pH and temperature).

B. INTRODUCTION

Allochthonous leaf litter inputs represent a valuable source of energy for stream ecosystems, especially in small, forested streams. In their classic study, Fisher and Likens (1973) showed approximately 99% of the energy available within their study stream at Hubbard Brook Experimental Forest (West Thornton, NH) consisted of allochthonous inputs. The breakdown of this allochthonous leaf litter, as defined by Hieber and Gessner (2002), is 'the combined result of physical and biological mineralization and transformation processes'. Leaf

breakdown consists of several phases including leaching, microbial conditioning, and fragmentation (by both physical abrasion and macroinvertebrate shredding activity) (Abelho, 2001; Boulton and Boon, 1991; Petersen and Cummins, 1974; Webster and Benfield, 1986). A variety of instream factors (both abiotic and biotic) have been shown to influence the rate of leaf breakdown, including leaf chemistry, macroinvertebrate abundance, dissolved nutrients, pH, dissolved oxygen, water temperature, and sedimentation (Sponseller and Benfield, 2001; Webster and Benfield, 1986).

Disturbance, both natural and anthropogenic, plays a major role in stream ecology and has been shown to directly and indirectly affect stream ecosystems (Resh *et al.*, 1988; Maloney and Weller, 2011). Anthropogenic disturbance is defined as "any human-mediated event or activity that is virtually unknown in natural systems in terms of type, frequency, intensity, duration, spatial extent, or predictability over the last century" (Naiman *et al.*, 2005). Anthropogenic disturbance can result from many land use activities, including but not limited to acid mining, agricultural practices, timber harvesting, and urbanization. According to the U.S. Census Bureau, the U.S. population is expected to increase by approximately 21% in the next twenty years (U.S. Census, 2008). Increases in human population size of this magnitude will greatly increase the occurrence of human-mediated disturbance in our natural ecosystems.

Human-mediated changes to the landscape can alter geomorphic processes and destabilize existing channel shapes and are often associated with above normal sedimentation rates via increased erosion and deposition (Maloney

125

et al., 2005). This increased sedimentation can alter or degrade many instream variables, such as stream habitat stability, as well as affect the structure of stream food webs (Allan, 2004; Downes *et al*., 2006; Henley *et al*., 2000). Instream environmental measures, similar to those that affect leaf breakdown, are greatly affected by sedimentation. Stream water temperature, turbidity and dissolved oxygen available in stream water are a few of the most notable factors affected (for review see Ryan, 1991; Lemly, 1982). Specifically with regard to aquatic invertebrates, sedimentation can affect population size and assemblage structure in multiple ways. Aquatic invertebrate density and diversity are directly related to substrate diversity (Reice, 1974; Gore, 1985). Sedimentation can reduce habitat availability for some invertebrates, making them more susceptible to predation (Newcombe and Macdonald, 1991). Aside from its effects on habitat availability, sediment can also affect certain invertebrate functional feeding groups, altering population size through its effects on primary production as well as by clogging feeding structures (Newcombe and Macdonald, 1991; Lemly, 1982).

With regard to the overall process of leaf breakdown, sedimentation has been shown to be capable of either increasing or decreasing the rate of breakdown, likely due to physical abrasion or burial, respectively (Benfield *et al*., 2001; Webster and Waide, 1982). Sponseller and Benfield (2001) found leaf breakdown rate to be positively correlated with substrate particle size. In this sense, smaller sediment likely buries leaf litter and reduces leaf breakdown, whereas larger sediment particles increase abrasion thus speeding up leaf

breakdown. Aside from burial, sedimentation and its subsequent effects on instream habitat can alter secondary producer composition, which can also lead to decreased breakdown rates (Sponseller and Benfield, 2001). These interactive effects between physical factors (i.e. sedimentation) and stream organisms can lead to drastic alterations in leaf breakdown and energy release.

Previous studies have shown decomposing leaf litter to be dominated by Gram-negative bacteria, including *Proteobacteria* (particularly the classes β- and γ-), *Actinobacteria*, as well as members of the *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) group (Das et al., 2006; Suberkropp and Klug, 1976). Changes in many environmental factors (e.g. nutrient levels, temperature, pH) have been shown to affect bacterial assemblages (Hill *et al.*, 2000; Witkamp, 1963 and 1966). These are also factors commonly altered via anthropogenic disturbance. In a broad sense, many of the bacteria previously found to be associated with leaf litter are also commonly found in aquatic sediments and terrestrial soils. Bacterial taxa found to be sediment-associated in the substrata of streams include many *Proteobacteria* (α-, β-, and γ-) and members of the CFB group (Santmire and Leff, 2007). In terrestrial environments, common soil bacteria include members of the phyla *Acidobacteria*, *Verrucomicrobia*, *Firmicutes*, *Gemmatimonadetes*, *Actinobacteria*, and *Proteobacteria* (specifically α-, β-, and γ-) (Joseph *et al.*, 2003). Although studies have explored common bacterial taxa in both aquatic and terrestrial environments, little is known regarding the effects of sedimentation on leaf litter-associated microbial community composition.

Given the role microbes play in the critical process of leaf breakdown, and the known effects of sedimentation on instream environmental conditions, as well as their potential effects on microbial communities, it is important to investigate the effects of sedimentation on leaf litter-associated microbial communities. The purpose of this study was specifically to examine the effects of sediment disturbance on bacterial assemblages in leaf packs during leaf breakdown. We hypothesized that increased amounts of sedimentation would both decrease the rate of leaf breakdown and decrease leaf litter-associated bacterial assemblage similarity. In addition, we hypothesized that this effect of sediment disturbance on leaf pack bacterial assemblage similarity would be the same regardless of leaf species.

C. METHODS

1. Study sites

This study was conducted in west-central Georgia at Fort Benning Military Installation (FBMI). FBMI occurs within the Sand Hills subecoregion of the Southeastern Plains ecoregion, south of the Fall Line. At FBMI, a combination of military training and forest management within military compartments has led to disturbance of upland terrestrial vegetation, underlying soil and, in turn, alterations in stream physicochemistry, organic matter abundance, and aquatic biota (Houser *et al.*, 2006; Maloney, Mulholland and Feminella, 2005; Maloney and Feminella, 2006). Within FBMI, I conducted a disturbance study in 6 streams (Kings Mill Creek tributary, Lois Creek, Bonham tributary, and 3 Sally Branch

tributaries, Table 5.1). All streams were second order except two (a first order Sally Branch tributary in compartment F1 and a third order Sally Branch Tributary in compartment F3). The study streams were low gradient with sandy substrate (mean particle size 0.56-0.89 mm) (Maloney *et al.*, 2005) and intact riparian canopy (Houser *et al.*, 2005; Maloney and Feminella, 2006).

To quantify the effects of stream disturbance on leaf breakdown, this study was conducted in 6 streams, 3 of which occur in highly disturbed watersheds (BC, SB2, SB3), with stream channels that show high sediment disturbance and low biotic integrity, and 3 streams in relatively less-disturbed watersheds (KM, LC, SB1) with correspondingly lower sediment disturbance and higher biotic integrity. Study streams were selected based on their disturbance intensity level, measured as the percentage of the watershed occurring as bare ground and road cover (Maloney *et al.*, 2005).

2. Experimental design

An *in situ* litter decomposition experiment was conducted using leaf species that were common riparian species at FBMI (Lockaby *et al.*, 2005). The leaf species included *Acer rubrum* (red maple) and *Quercus nigra* (water oak). These leaf species were chosen because they span a range of breakdown rates, with red maple having a medium breakdown rate (k=0.005-0.010) and water oak a relatively low breakdown rate (k<0.005) (Webster and Benfield, 1986).

There were 5 collection dates (days 0, 8, 16, 32, and 64) ranging from January to March 2007. The 3 streams from each disturbance treatment (low- vs. high-disturbance) were used as replicates and sampled for leaf litter bags on

each date. Each block consisted of one run habitat containing 4 replicates of each leaf species with each block sampled on 1 of the 5 dates; blocks were chosen randomly during the 64-d study.

Artificial leaf packs held within mesh bags (0.1524 m x 0.3048 m) and placed *in situ* were used, as this is a common method for studying leaf breakdown in streams (Boulton and Boon, 1991). Leaves were collected from a single tree of each species during fall 2006 (December-January) using tarps strung below trees to accumulate abscissed leaves. Leaves were air-dried in a sterile Class II biosafety cabinet to a constant mass, weighed into 4-g aliquots, and then placed into sterilized mesh bags until deployed. Mesh bags of leaf packs had coarse (6.35 mm) mesh on one side to allow colonization of macroinvertebrates and a smaller (3.175-mm) mesh on the other side to reduce loss of litter particles from inside the bag during incubation. Once filled, mesh bags were sewn closed with nylon and then anchored in the stream with rebar. For each leaf species day 0 samples were taken by briefly dipping packs into the stream water and then removing and returning them to the laboratory to quantify handling loss (Petersen and Cummins, 1974).

On the specified collection date, we removed each leaf pack from the block, placed it in a Ziploc bag, and returned it on ice to the laboratory. A 2-leaf subsample was taken from each leaf pack for microbial processing, and the remaining leaves were used for determination of breakdown rate (below). The leaf subsample was ground in liquid N_2 and stored it at $-80°C$ until processed for microbial community characterization (below).

The remaining leaf litter was rinsed to remove any benthic macroinvertebrates and sediment. All macroinvertebrates were stored in 70% ethanol and then sorted, measured (to the nearest mm), and identified to genus when possible (except for Oligochaeta and Acari, which were identified to subclass). Given their major role in leaf litter fragmentation, shredding macroinvertebrates were identified according to Merritt and Cummins (1996). Mean abundance of macroinvertebrates and shredders, as well as total macroinvertebrate and shredder density (ind./g AFDM remaining) was calculated for each leaf species/disturbance combination at each time point. The percentages of taxa in the family Chironomidae and the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT), were estimated due to both their general sedimentation tolerance (Shaw and Richardson, 2001) and disturbance sensitivity (Barbour *et al.*, 1999), respectively. In addition, we estimated total taxa richness, Shannon diversity (*H'*), and total biomass (Benke, 1999).

To determine breakdown rates, the remaining leaf litter was then dried to a constant mass at $60^{o}C$, weighed, and then combusted in a muffle furnace at $550^{o}C$ for 2 h. The ashed residue was reweighed and subtracted from the pre-combusted dry mass for determination of ash-free dry mass (AFDM). Breakdown rates were calculated using an exponential decay model (Petersen and Cummins, 19974) as the slope of the regression line of ln(% AFDM remaining) vs time. The amount of sediment from each pack was quantified from all leaf packs, excluding those from KM, which were from a separate study where no sediment was recorded. All sediment rinsed from leaf litter and remaining after sorting of

macroinvertebrates was dried to a constant mass at 60°C, weighed, and combusted in a muffle furnace at 550°C for 2 h to move all organic material. Following this, the sediment was weighed and recorded as the amount of sediment in each leaf pack.

To characterize variation in physicochemical conditions known to affect breakdown (Webster and Benfield 1986; Dangles *et al.,* 2004), we quantified streamwater temperature, pH, depth, and current velocity. Water temperature was measured hourly with HOBO Temp data loggers. Streamwater pH was measured using a Thermo Orion Model 420. Differences in depth and current velocity were quantified to assess variation in leaf breakdown and microbial communities attributable to depth or velocity differences. Leaf pack depth was measured using a meter stick placed at the top center of each leaf pack, and a Marsh-McBirney Flowmate current meter was used to measure current velocity within each leaf pack. Current velocity inside each leaf pack was measured by positioning an empty "dummy" bag over the probe placed immediately upstream of each leaf pack.

3. Bacterial assemblage characterization

3.1 DNA extraction for molecular analyses. We used leaf subsamples for 2 separate molecular analyses of bacterial communities: 1) ribosomal intergenic spacer analysis (RISA) and 2) 454 pyrosequencing. For these procedures, we isolated genomic DNA from 0.10-g leaf litter using a Qiagen genomic DNA extraction kit (Qiagen, Valencia, CA, USA). DNA was purified using a cetyltrimethylammonium bromide (CTAB) extraction procedure (Ausubel, 1994).

In some cases, extracted DNA was not sufficiently pure to serve as template for PCR. For those samples, we conducted an additional round of genomic DNA purification using a combination of 80% formamide and 1M NaCl treatment to provide PCR-ready genomic DNA template (see Chapter II). This formamide purification step has been tested with DNA extracted from many different environments and has not been observed to result in any loss of DNA or corresponding loss of diversity as assessed by DGGE. If this method was deemed necessary by the lack of PCR amplification using DNA templates derived from commercial kit extraction, then the formamide purification method was consistently applied to all samples from that sampling date.

3.2 RISA analysis of ITS regions. RISA analysis was accomplished by PCR amplification of bacterial internal transcribed spacer (ITS) regions and separating polymorphic ITS amplicons within a polyacrylamide gel matrix. PCR was conducted within a volume of 10 µL containing GoGreen Master Mix (Promega; Madison, WI), 1x bovine serum albumin (BSA), nuclease free water, primers, and approx. 1-5 ng genomic DNA template, quantified spectrophotometrically with a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Primers used for these reactions were the universal bacterial primers IRDYE 800-labeled ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') (Cardinale *et al.*, 2004) and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al.*, 2004) at a final concentration of 0.20 µM. This primer set has been shown to not be as susceptible as other primers to known PCR biases such as those due to substrate reannealing (Suzuki and Giovannoni, 1996) and preferential amplification of shorter DNA

templates (Cardinale *et al.*, 2004). Some studies have found that a small

percentage of 16S rDNA amplicons, derived from using universal 16S-rDNA

bacterial primers with DNA template from leaves, corresponded to plant 16S

rDNA sequences (Kim *et al.*, 2010; Saito *et al.*, 2007). However, the

ITSF/ITSReub primers used in this study for ITS region amplification have not

shown this problem (Cubaka Kabagale *et al.*, 2010). Amplification was done

according to the method of Fisher and Triplett (1999), as follows: reaction

mixtures were held at 94°C for 2 min, followed by 30 cycles of amplification at

94°C for 15 s, 55°C for 15 s, and 72°C for 45 s and a final extension of 72°C for

2 min. PCR products were verified on a 1% agarose gel stained with ethidium

bromide. Following verification of product yield and size, we separated amplicons

in a 5.5% polyacrylamide gel matrix and images were recorded using a Li-Cor

4300 (Li-Cor Inc., Lincoln, NE, USA).

3.3 Bar-coded pyrosequencing of bacterial assemblages. Twelve red maple

samples were selected for further pyrosequencing of their bacterial assemblages.

These 12 samples represented the later time points (32 and 64 days), and for

each time point we used two replicates from the highest (SB3) and lowest (LC)

disturbance sites and one replicate from the second highest (SB2) and lowest

(KM) disturbance sites. A 457-bp region of the 16S rRNA gene was amplified that

included the hypervariable regions V3 and V4, using a fusion primer set found to

be suitable for classification of 16S rRNA genes from complex microbiomes

(Nossa *et al.,* 2008). The forward primer (5'-

CGTATCGCCTCCCTCGCGCCATCAG-NNNNNNNNNN-

GGAGGCAGCAGTRRGGAAT-3') contained the Roche adaptor A, a unique 10-bp MID-barcode used to tag each PCR product (designated by NNNNNNNNNN; see Table 5.2), and the bacterial primer 347F (Nossa *et al.,* 2008). The reverse primer (5'–CTATGCGCCTTGCCAGCCCGCTCAG-CTACCRGGGTATCTAATCC-3') contained the Roche adaptor B, and the bacterial primer 803R (Nossa *et al.,* 2008). PCRs consisted of 2 µl of each 12.5 µM forward and reverse fusion primer, 2 µl of BSA (100X), 5 µl of template DNA, and 25 µl *PfuUltra* Hotstart PCR Master Mix (2X) (Agilent Technologies; Wilmington, DE, USA) and were adjusted to a final volume of 50 µl using nuclease free water. Samples were initially denatured at 95°C for 1 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A final extension of 10 min at 72°C was added to ensure complete amplicons extension. Following verification of PCR products on a 1% agarose gel, PCR products were purified using an ethanol precipitation, and DNA concentration was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Purified PCR products were then diluted to equimolar concentrations (16ng/µl), pooled, and sequenced using 454 GS FLX Titanium chemistry (Engencore; Columbia, SC, USA).

4. Data analyses

Physicochemical conditions were compared between low- and high-sediment disturbance sites using 1-way ANOVAs (Zar, 1999). When necessary, physicochemical values (particularly sediment) were log transformed in order to achieve a normal distribution. Breakdown rates were compared for each leaf

species between low- and high-sediment disturbance sites using 1-way ANOVAs (Zar, 1999). Macroinvertebrate metrics were not normally distributed and were compared between low- and high-sediment disturbance sites for each leaf species using a Kruskal-Wallis test (Kruskal and Wallis, 1952). Correlations between macroinvertebrate metrics and the amount of sediment found in leaf packs were tested using Spearman's rank correlation for all macroinvertebrate metrics that showed a significant difference between high- and low-sediment disturbance sites for multiple time points. RISA gel images were analyzed using BioNumerics Software v5.0 (Applied Maths, Sint-Martens-Latem, Belgium). Bands were defined relative to the highest band density on that pattern, where all bands, with a density >10% of the highest band density, were selected and used to create a presence-absence matrix for further analysis. Nonmetric multi-dimensional scaling (NMDS) based on a Bray-Curtis dissimilarity index (Bray and Curtis, 1957) and three dimensions was used to visualize differences in RISA profiles of bacterial assemblages, and an Analysis of Similarity (ANOSIM) (Clarke, 1993) was used to determine if low- and high-sediment disturbance profiles for each leaf species were significantly different.

Pyrosequencing data was processed using the software pipeline Quantitative Insights Into Microbial Ecology (QIIME) as described (Caporaso *et al.*, 2010). Prior to analysis in QIIME, sequences were trimmed using the CLC Genomics Workbench (CLC Bio), and only sequences with a quality score of at least 25, containing no ambiguous nucleotides or mismatches in the primer sequence, and a minimum length of 200bp were used for further analyses.

Sequences were imported into QIIME, sorted based on their respective bar codes and denoised using Denoiser (version 0.91) (Reeder and Knight, 2010). Sequences were grouped into Operational Taxonomic Units (OTUs) using the "uclust" method and a similarity threshold of 97%. These individual OTUs were then classified using the RDP classifier (>80% confidence) (Wang *et al.*, 2007) and aligned using PyNAST (Caporaso *et al.*, 2009).

To estimate diversity, these sequences were rarefied and analyzed at the same level of surveying effort (185 sequences per sample). Alpha diversity was estimated within each sample and included measuring observed species (OTUs), Shannon-Wiener diversity (*H'),* and estimated species richness (Chao1). Presence and absence of individual OTUs among samples (beta diversity) was compared using a pairwise, unweighted UniFrac distance matrix and visualized in two dimensions using NMDS based on a Bray-Curtis dissimilarity index (Bray and Curtis, 1957). An ANOSIM (Clarke, 1993) was used to determine if low- and high-sediment disturbance bacterial assemblage compositions were significantly different. Differences between the relative abundance of specific bacterial taxonomic groups in low- vs. high-sediment disturbance sites at individual times were tested for using 1-way ANOVAs (Zar, 1999). Redundancy analysis (RDA) was used, following a Hellinger transformation, to see if the physicochemical variables measured explained a significant portion of the variation observed in pyrosequenced bacterial assemblage composition (Legendre and Gallagher, 2001). Forward stepwise regression was then used to determine which physicochemical variables specifically explained the most variation. Correlations

were also tested for among environmental variables and between environmental variables and relative abundance of bacterial taxa using Pearson's correlation. For all statistical tests, unless otherwise noted, a significance level of α=0.05 was used to determine significance. All statistical computations were completed using SigmaPlot for Windows (version 12), MINITAB (version 15), and R Statistical Software (including the packfor and vegan packages) (version 2.13.1).

D. RESULTS

1. Physicochemical conditions

Over the 64-d study (January to March) average water temperature was coldest in LC (10.03°C) and warmest in SB2 (10.54°C) (Table 5.3). Overall, average water temperature in low-sediment disturbance streams (10.08°C) was significantly cooler than high-sediment disturbance streams (10.36°C) (p=0.004). Streamwater pH ranged from a low of 3.92 to a high of 5.31 (mean=4.49) (Table 3). The average depth of leaf packs between low- and high-sediment disturbance sites was not significantly different (p=0.249) (Table 5.3). Average current velocity measurements between low- and high-sediment disturbance sites were also not significantly different (p=0.416) (Table 5.3).

Sediment in leaf packs varied significantly by site (p<0.001) and steadily increased over the 64-day study period. Leaf packs from SB1, although considered a low-sediment disturbance site based on calculated disturbance intensity, had the highest mean amount of sediment (153.8 g). We believe the increased sedimentation in this stream is likely due to a legacy effect from past

land use practices at Fort Benning. For instance, site data from 1944 indicates the percent bare ground and road cover to be approx. 25.7% versus its recent measure of 8.4% (K.O. Maloney, unpubl. data). In addition, BC, which was considered a high-sediment disturbance site based on its calculated disturbance intensity had very little sediment within leaf packs (mean=7.70g). This stream has been noted to behave differently than predicted for its catchment disturbance level in previous studies of these sites (Mulholland *et al.*, 2005). It is believed that the presence of a broad forested floodplain bordering the catchment of this stream allows this stream to remain fairly undisturbed, despite having a disturbed catchment (Mulholland *et al.*, 2005). Leaf packs from KM were from a separate study, and at the time, no sediment data were recorded. However, from personal observation the amount of sediment in these packs was minimal and comparable to the amount found in leaf packs from site LC. Leaf packs from SB3 (mean=95.50 g) and SB2 (mean=91.60 g) both contained large amounts of sediment, and LC leaf packs had the lowest mean amount of sediment (2.49 g).

2. Litter breakdown

Rates of leaf breakdown for red maple (mean k=0.021) were significantly faster than water oak (mean k=0.005) (p<0.001). Overall, the rate of breakdown for red maple (hereafter maple) in low-sediment disturbance sites was significantly faster than in high-sediment disturbance sites (p=0.005) with k=0.026 and k=0.016 in low- and high-sediment disturbance sites, respectively (Figure 5.1). Water oak (hereafter oak) breakdown rates were not significantly

different between oak leaves incubated in low- and high-sediment disturbance streams (p=0.879) with k=0.004 in both treatments (Figure 5.1).

3. Macroinvertebrates

For both leaf species, mean taxa richness steadily increased throughout the study and was significantly higher in low-disturbance leaf packs than high-disturbance for both leaf species (maple p=0.014 and oak p=0.004) (see Table 5.4). Shannon's diversity and macroinvertebrate abundance, density, and biomass were significantly higher in low-disturbance leaf packs for both leaf species (see Table 5.4). Shredder abundance and density were not significantly different between low- and high-disturbance streams for maple leaf packs. However, oak leaf packs in low-disturbance streams contained significantly higher shredder abundance and density. In general, both leaf species showed higher shredder abundance and density in low-disturbance streams than high-disturbance streams, but this difference was only significant for oak leaf packs (p=0.012). Taxa richness and Shannon's diversity estimates were significantly correlated to the amount of sediment within a leaf pack for both maple and oak on day 64 (see Figures 5.2 and 5.3). The percentage of macroinvertebrates from the family Chironomidae and the orders Ephemeroptera, Plecoptera, and Trichoptera was not significantly different between high- and low-disturbance stream leaf packs for either leaf species (see Table 5.4).

4. Bacterial assemblage characterization

NMDS of RISA profiles using the Bray-Curtis dissimilarity index and 3 dimensions revealed separation of high- and low-sediment disturbance leaf pack

RISA profiles following an approx. 1-month instream incubation (days 32 and 64) for both leaf species (Figures 5.4 and 5.5; see Figures 5.4 and 5.5 for plots of first 2 dimensions and stress levels). Analysis of similarity (ANOSIM) between low- and high-sediment disturbance leaf pack RISA profiles revealed the separation observed in the NMDS plots at days 32 and 64 to be significant for both maple (day 32, global R=0.23, p=0.016; day 64, global R=0.31, p=0.003) and oak (day 32, global R=0.74, p=0.001; day 64, global R=0.23, p=0.003). There was no significant separation of RISA profiles between low- and high-sediment disturbance leaf packs for maple or oak on day 8 (ANOSIM, p= 0.076 and p=0.052, respectively) or day 16 (ANOSIM, p=0.477 and p=0.241, respectively).

Given the significant difference in RISA profiles between low- and high-sediment disturbance leaf packs at days 32 and 64, these time points were further selected for pyrosequencing and analysis of their bacterial assemblages. Since a significant difference in breakdown rate was only observed for maple leaves in low- vs. high-sediment disturbance sites, a subset of twelve maple samples from days 32 and 64 were used for this analysis. These samples included 3 low- and 3 high-sediment disturbance samples from each time point (days 32 and 64). From these analyses, we were able to obtain sufficient high-quality sequences from 10 samples, 6 from day 32 (3 low and 3 high) and 4 from day 64 (2 low and 2 high). A total of 5694 sequences were obtained for classification with a mean of approx. 570 classifiable sequences per sample (range 185-1178). In general, after 32 days instream, low-sediment disturbance

leaf pack bacterial assemblages had more OTUs and higher diversity than high-sediment disturbance leaf packs (Table 5.5), and the reverse of this trend was observed after 64 days insteam (Table 5.5). Overall, these differences were not statistically significant for either time point. Pairwise, unweighted UniFrac distances were visualized using NMDS (k=2; stress=0.081) (see Figure 5.6) and overall showed significant separation between low- and high-sediment disturbance samples (ANOSIM; global R=0.52, p=0.019).

Summaries of the bacterial assemblage composition for each day x disturbance treatment are depicted in Figures 5.7 and 5.8. Low-sediment disturbance leaf packs on day 32 contained many bacterial phyla including taxa that affiliated with the phyla *Proteobacteria* (77.1%), *Acidobacteria* (12.6%), CFB group (4.5%), *Actinobacteria* (1.5%), and *Firmicutes* (0.8%). High-sediment disturbance leaf packs on day 32 also contained *Proteobacteria* (54.8%), *Acidobacteria* (3.1%), CFB group (8.9%), and *Actinobacteria* (3.6%). However, these samples had a significantly higher proportion of taxa affiliated with the phylum *Firmicutes* (25.1%; p=0.046), 93% of which were from the class Clostridia, mostly of the family Clostridiaceae. Low- and high-sediment disturbance leaf packs after 32 days contained similar proportions of taxa that affiliated with the phyla α-*Proteobacteria* (41.4%, 56.5%) and γ-*Proteobacteria* (16.5%, 16.5%). Low-sediment disturbance leaf packs contained a higher proportion of β-*Proteobacteria* (38.6% vs. 19.0%; p=0.755) than high-sediment disturbance leaf packs, and high-sediment disturbance leaf packs contained a higher, although not significant (p=0.854), proportion of δ-*Proteobacteria* (7.4%

vs. 2.9%) than low-sediment disturbance leaf packs. Approximately three-fourths (73.9%) of δ-*Proteobacteria* in high-sediment disturbance leaf packs came from the family Geobacteraceae.

Following 64 days instream incubation, low-sediment disturbance leaf packs contained taxa that affiliated with the phyla *Proteobacteria* (79.5%), *Acidobacteria* (10.0%), CFB group (5.2%), *Firmicutes* (1.8%), and *Actinobacteria* (0.9%). The taxa that affiliated within the phylum *Proteobacteria* could be further classified at the class level with 46.0% α-*Proteobacteria*, 45.2% β-*Proteobacteria*, 5.1% γ-*Proteobacteria*, and 3.2% δ-*Proteobacteria.* After 64 days in stream, high-sediment disturbance leaf packs contained similar proportions of taxa that affiliated with the phyla *Proteobacteria* (74.9%), *Actinobacteria* (3.1%), and CFB group (3.7%) to those of low-sediment disturbance leaf packs. The proportion of taxa within the phylum *Firmicutes* dropped to 4.0%, compared to 25.1% after 32 days (p=0.116). The *Proteobacteria* taxa identified within the high-sediment disturbance leaf packs*,* similar to the low-sediment disturbance leaf packs, consisted mostly of α-*Proteobacteria* (34.7%), β-*Proteobacteria* (41.6%), and γ-*Proteobacteria* (5.8%) but had a significantly higher proportion of representatives from the class δ-*Proteobacteria* than low-sediment disturbance leaf packs (17.1%; p=0.040). Most of these were from the suborders Sorangineae (25.8%) and Cystobacterineae (24.0%) and the family Geobacteraceae (25.6%).

Overall, low-sediment disturbance leaf packs contained a significantly higher proportion of taxa that affiliated with the phylum *Acidobacteria* than high-

sediment disturbance leaf packs (p=0.041). The relative abundance of

Acidobacteria taxa in leaf packs was significantly negatively correlated to

streamwater pH at the α=0.10 level (p=0.058, r=-0.61). High-sediment

disturbance leaf packs tended to have a higher relative abundance of *Firmicutes*

taxa than low-sediment disturbance leaf packs, and the relative abundance of

Firmicutes taxa was significantly positively correlated to the amount of sediment

in leaf packs (p=0.049, r=0.67). For both low- and high-sediment disturbance leaf

packs on day 64, there was a decrease (p=0.338), in the proportion of

representatives from γ-*Proteobacteria*, particularly in the family

Pseudomonadaceae, which was concomitantly associated with an increase in

the proportion of α- and β-*Proteobacteria* in low-sediment and β- and δ-

Proteobacteria in high-sediment disturbance leaf packs. For both disturbance

and time treatments, common α-*Proteobacteria* taxa came from the orders

Caulobacterales, Rhizobiales, Rhodospirillales, and Sphingomonadales.

Common β-*Proteobacteria* taxa for both disturbance and time treatments were

members of the order Burkholderiales including the families Oxalobacteraceae

and Comamonadaceae.

For the leaf pack bacterial assemblages that were assessed using

pyrosequencing, a global RDA test using all physicochemical variables was

significant (p=0.017). Following this, forward stepwise regression was performed

using six physicochemical variables as explanatory variables (site disturbance

intensity, amount of sediment in pack, water temperature, pH, depth, and current

velocity). This analysis resulted in the selection of pH (p=0.002) as the significant

explanatory variable, explaining 31.17% of the variance in bacterial community composition. A Mantel correlation between bacterial assemblage distance and the environmental distance of all six physicochemical variables was significant (p=0.003; r=0.47). If only pH was examined in this manner, the correlation between bacterial assemblage and the environmental distance matrix was significant (p=0.002, r=0.72). For the sites and samples examined via pyrosequencing, streamwater pH was also significantly correlated to disturbance intensity (p<0.015; r=0.77), and disturbance intensity was significantly correlated to sediment (p=0.035; r=0.70).

E. DISCUSSION

In this study the breakdown of maple litter, a medium-degrading leaf species, was significantly decreased in the presence of high-sediment disturbance. Given the smaller particle size of these streams (0.56-0.89 mm) and the increased sediment in high-sediment disturbance leaf packs, it is possible that the decreased breakdown of maple was due to burial by sediment, which has been suggested in other leaf breakdown studies (Bunn, 1988; Benfield *et al*., 2001). The lack of a similar effect of increased sedimentation on oak breakdown could be due to its chemical structure that results in a slower breakdown rate. It is possible that a greater effect of sedimentation would have been observed for oak leaf litter if this study had been conducted for a longer period of time that captured the full range of breakdown for oak.

Increased sediment disturbance and sediment within leaf packs led to was associated with decreases in many macroinvertebrate metrics (taxa richness, macroinvertebrate abundance and density, Shannon's diversity, and biomass), which also could have contributed to decreased leaf breakdown, especially given the large role of macroinvertebrates in leaf breakdown (Cummins *et al.*, 1973). Similar to previous studies involving sediment disturbance (Hagen *et al.*, 2006; Jones *et al.*, 2011), the leaf packs incubated in high-sediment disturbance sites had decreased macroinvertebrate abundance and density. And, although not significant for both leaf species, there was an overall trend towards decreased shredder abundance and density in high-sediment disturbance leaf packs. As the amount of sediment in leaf packs increased, decreases in macroinvertebrate taxa richness and diversity (*H'*) were observed, a trend that has also generally been observed in streams with increased sedimentation (Jones *et al.*, 2011). Taken together with the decrease in leaf breakdown, the macroinvertebrate assemblage characteristics observed in this study indicate that our low- and high-sediment disturbance sites were similar to those typically observed by other sediment disturbance studies (Jones *et al.*, 2011; Maloney *et al.*, 2011; Sponseller and Benfield, 2001).

Effects of increased sediment disturbance on leaf litter bacterial assemblages were observed following one month of instream incubation for both medium- (maple) and slow-degrading (oak) leaf species where bacterial assemblage similarity between low- and high-sediment disturbance leaf packs was decreased. This suggests that sediment disturbance can alter bacterial

assemblage composition during the later periods of bacterial succession on leaf litter. Maple leaves demonstrated an effect of high-sediment disturbance on both leaf breakdown rate and the associated bacterial assemblage, and both later time points were selected for bacterial assemblage characterization via pyrosequencing. Although no significant differences were found in measures of alpha diversity between low- and high-sediment disturbance bacterial assemblages, significant differences in bacterial assemblage composition (beta diversity) were observed between low- and high-sediment disturbance sites, further indicating that high-sediment disturbance alters bacterial assemblage composition during later stages of breakdown. Low-sediment disturbance leaf packs shared similarity with findings of previous studies, being dominated by mostly gram-negative bacteria affiliated with the *Proteobacteria* and *Acidobacteria* phyla (Das *et al*., 2006; Suberkropp and Klug, 1976)*.*

Both disturbance treatments revealed decreases over time in the relative abundance of taxa affiliated with the γ-*Proteobacteria* (particularly *Pseudomonas* spp.), with members of this genus often described as being opportunistic, r-strategists due to their relatively fast growth (Juteau *et al*., 1999; Yang and Lou, 2011). Along with this decrease, there were increases in the relative abundance of many α- and δ-*Proteobacteria* taxa, with all common orders and suborders found (e.g. Caulobacterales, Rhizobiales, Rhodospirillales, and Sphingomonadales, Sorangineae, Cystobacterineae, Geobacteraceae) being efficient users of environmental resources and typically considered K-strategists (as compared to other bacteria such as *Pseudomonas* spp.) due to their slower

colonization and growth rates and higher energy investment in maintenance rather than reproduction (Mikkonen, 2011; Bastian *et al*., 2009). The K-strategists colonizing leaf packs differed in relative abundance between low- and high-sediment disturbance leaf packs. Low-sediment disturbance leaf packs saw increases in α-*Proteobacteria* orders, whereas high-sediment disturbance leaf packs had increases in δ-*Proteobacteria* suborders.

High-sediment disturbance leaf packs had a significantly different bacterial assemblage composition from that of low-sediment disturbance leaf packs following just one month of instream incubation. These high-sediment disturbance leaf packs had high relative abundances of taxa within the phyla *Firmicutes* (93% from the class *Clostridia*, mostly of the family Clostridiaceae), which are endospore-forming heterotrophs and obligate anaerobes (Griebler and Lueders, 2009), and probably K-strategist taxa within the δ-*Proteobacteria* (*Geobacter* spp. [order Desulfuromonadales] and members of the suborders Sorangineae and Cystobacterineae [both from the order Myxococcales]). All of these taxa are capable of surviving either as endospores or as cells with lower metabolic activity in environments where nutrients are exhausted and oxygen is absent (Garrity *et al.*, 2005). These results point towards a shift under high-sediment disturbance conditions to a bacterial assemblage that is better capable of withstanding harsh environmental conditions, particularly low oxygen. This effect of sedimentation on oxygen availability has also been postulated by observations made by Sponseller and Benfield (2001). An alteration in bacterial assemblage composition via environmental conditions could consequently alter

148

the bacterial metabolic activity within leaf packs. Navel *et al.* (2010), in an *in vitro* study, showed fine sediment deposition led to decreases with depth in both oxygen concentration and the percentage of active bacteria, leading to a 30% decrease in leaf breakdown in their study. However, it is not known in this study exactly what percentage of decreased breakdown was due to altered bacterial assemblage composition alone, given the differences in macroinvertebrate metrics and burial of leaf packs in high-sediment disturbance leaf packs both of which can drastically impact leaf breakdown.

A major difference between high- and low-sediment disturbance leaf pack bacterial assemblages that occurred regardless of time was an increased relative abundance of taxa affiliated with the phylum *Acidobacteria* in low-sediment disturbance leaf packs. This high relative abundance of *Acidobacteria* in low-sediment disturbance leaf packs is surprising given that *Acidobacteria* are commonly found in terrestrial sediments (Rappe and Giovannoni, 2003) and would therefore be predicted to be higher in high-sediment disturbance leaf packs. However, it is possible that the relative abundance of *Acidobacteria* taxa is increased in low-sediment disturbance leaf packs due to the decreased pH found at low-sediment disturbance sites, especially since *Acidobacteria* taxa have been shown to thrive in lower pH environments (Jones *et al.*, 2009; Sait *et al.*, 2006), and were also significantly negatively correlated with streamwater pH in this study. Previous studies on these sites have also found a significant correlation between streamwater pH and disturbance intensity via land use and increased sedimentation (Houser *et al.*, 2006).

The results of this study agree with the r/K selection continuum (MacArthur and Wilson, 1967; Andrews and Harris, 1986) with observed decreases over time in *Pseudomonas* spp. (r-strategists) and increases in many taxa considered to be K-strategists and endospore-formers. For bacterial assemblages in high-sediment leaf packs, changes in the instream environment associated with high-sediment disturbance (i.e. increased pH, leaf pack burial via sedimentation) was associated with a shift towards a leaf pack bacterial assemblage dominated by taxa capable of surviving under harsher environmental conditions (i.e. low oxygen, high pH) (Figure 5.9). The results of this study illustrate that high-sediment disturbance can potentially lead to long-term effects on leaf litter bacterial assemblage composition through its effects on the instream environment, particularly pH.

F. LITERATURE CITED

Abelho, M. 2001. From litterfall to breakdown in streams: a review. *The Scientific World Journal,* 1, 656-680.

Al Shaw, E. and Richardson, J.S. 2001. Direct and indirect effects of sediment pulse duration on stream invertebrate assemblages and rainbow trout Oncorhynchus mykiss. growth and survival. *Canadian Journal of Fisheries and Aquatic Sciences,* 58, 2213-2221.

Allan, J.D. 2004. Landscapes and riverscapes: the influence of land use on stream ecosystems. *Annual Review of Ecology, Evolution, and Systematics,* 35, 257-284.

Andrews, J.H. and Harris, R.F. 2000. The ecology and biogeography of microorganisms on plant surfaces. *Annual Review of Phytopathology,* 38, 145-180.

Ausubel, F.M. 1994. Preparation of plant DNA using CTAB. *Current Protocols in Molecular Biology,* Suppl 27, 2.3-2.3.7.

Barbour, M.T., Gerritsen, J., Snyder, B.D. and Stribling, J.B. 1999. Rapid bioassessment protocols for use in streams and wadeable rivers. *USEPA, Washington*.

Bastian, F., Bouziri, L., Nicolardot, B. and Ranjard, L. 2009. Impact of wheat straw decomposition on successional patterns of soil microbial community structure. *Soil Biology and Biochemistry,* 41, 262-275.

Benfield, E.F., Webster, J.R., Tank, J.L. and Hutchens, J.J. 2001. Long Term

    Patterns in Leaf Breakdown in Streams in Response to Watershed

    Logging. *International Review of Hydrobiology,* 86, 467-474.

Benke, A.C., Huryn, A.D., Smock, L.A. and Wallace, J.B. 1999. Length-mass

    relationships for freshwater macroinvertebrates in North America with

    particular reference to the southeastern United States. *Journal of the*

    North American Benthological Society, 18, 308-343.

Boulton, A.J. and Boon, P.I. 1991. A review of methodology used to measure leaf

    litter decomposition in lotic environments: time to turn over an old leaf?

    Marine and Freshwater Research, 42, 1-43.

Bray, J.R. and Curtis, J.T. 1957. An ordination of the upland forest communities

    of southern Wisconsin. *Ecological monographs,* 27, 325-349.

Bunn, S.E. 1988. Processing of leaf litter in two northern jarrah forest streams,

    Western Australia: II. The role of macroinvertebrates and the influence of

    soluble polyphenols and inorganic sediment. *Hydrobiologia,* 162, 211-223.

Caporaso, J.G., Bittinger, K., Bushman, F.D., Desantis, T.Z., Andersen, G.L. and

    Knight, R. 2009. PyNAST: a flexible tool for aligning sequences to a

    template alignment. *Bioinformatics,* 26, 266.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D.,

    Costello, E.K., Fierer, N., Peòa, A.G., Goodrich, J.K. and Gordon, J.I.

    2010. QIIME allows analysis of high-throughput community sequencing

    data. *Nature methods,* 7, 335-336.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A.M., Rizzi, A., Zanardini, E., Sorlini, C., Corselli, C. and Daffonchio, D. 2004. Comparison of Different Primer Sets for Use in Automated Ribosomal Intergenic Spacer Analysis of Complex Bacterial Communities. *Applied and Environmental Microbiology,* 70, 6147-6156.

Clarke, K. R. 1993. Non-parametric multivariate analysis of changes in community structure. *Australian Journal of Ecology* 18, 117-143.

Cubaka Kabagale, A., Cornu, B., Van Vliet, F., Meyer, C.L., Mergeay, M., Lumbu Simbi, J.B., Droogmans, L., Vander Wauven, C. and Verbruggen, N. 2010. Diversity of endophytic bacteria from the cuprophytes Haumaniastrum katangense and Crepidorhopalon tenuis. *Plant and soil,* 334, 461-474.

Cummins, K.W., Petersen, R.C., Howard, F.O., Wuycheck, J.C. and Holt, V.I. 1973. The utilization of leaf litter by stream detritivores. *Ecology,* 336-345.

Dangles, O., Gessner, M.O., Guerold, F. and Chauvet, E. 2004. Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. *Journal of Applied Ecology,* 41, 365-378.

Das, M., Royer, T.V. and Leff, L.G. 2006. Fungal, bacterial and actinomycete diversity on leaves decomposing in a stream. *Applied and Environmental Microbiology*, 73, 756-767.

Downes, B.J., Lake, P.S., Glaister, A. and Bond, N.R. 2006. Effects of sand sedimentation on the macroinvertebrate fauna of lowland streams: are the effects consistent? *Freshwater biology,* 51, 144-160.

Fisher, M.M. and Triplett, E.W. 1999. Automated Approach for Ribosomal
Intergenic Spacer Analysis of Microbial Diversity and Its Application to
Freshwater Bacterial Communities. *Applied and Environmental
Microbiology,* 65, 4630-4636.

Fisher, S.G. and Likens, G.E. 1973. Energy flow in Bear Brook, New Hampshire:
an integrative approach to stream ecosystem metabolism. *Ecological
monographs,* 43, 421-439.

Garrity, G.M., Brenner, D.J. and Krieg, N.R. 2005. *Bergey's manual of systematic
bacteriology,* Springer.

Gore, J.A. 1985. Mechanisms of colonization and habitat enhancement for
benthic macroinvertebrates in restored river channels. *The Restoration of
Rivers and Streams: Theories and Experience, Butterworth Publishers,
Boston Massachusetts. 1985. p 81-101, 7 fig, 80 ref.*

Griebler, C. and Lueders, T. 2009. Microbial biodiversity in groundwater
ecosystems. *Freshwater biology,* 54, 649-677.

Hagen, E.M., Webster, J.R. and Benfield, E.F. 2006. Are leaf breakdown rates a
useful measure of stream integrity along an agricultural landuse gradient?
Journal of the North American Benthological Society, 25, 330-343.

Henley, W.F., Patterson, M.A., Neves, R.J. and Lemly, A.D. 2000. Effects of
sedimentation and turbidity on lotic food webs: a concise review for natural
resource managers. *Reviews in Fisheries Science,* 8, 125-139.

Hieber, M. and Gessner, M.O. 2002. Contribution of stream detrivores, fungi, and
bacteria to leaf breakdown based on biomass estimates. *Ecology,* 83,
1026-1038.

Hill, B.H., Hall, R.K., Husby, P., Herlihy, A.T. and Dunne, M. 2000. Interregional
comparisons of sediment microbial respiration in streams. *Freshwater
biology,* 44, 213-222.

Houser, J.N., Mulholland, P.J. and Maloney, K.O. 2005. Catchment disturbance
and stream metabolism: patterns in ecosystem respiration and gross
primary production along a gradient of upland soil and vegetation
disturbance. *Journal of the North American Benthological Society,* 24,
538-552.

Houser, J.N., Mulholland, P.J. and Maloney, K.O. 2006. Upland disturbance
affects headwater stream nutrients and suspended sediments during
baseflow and stormflow. *Journal of Environmental Quality,* 35, 352-365.

Jones, J.I., Murphy, J.F., Collins, A.L., Sear, D.A., Naden, P.S. and Armitage,
P.D. 2011. The impact of fine sediment on macro invertebrates. *River
Research and Applications*. DOI: 10.1002/rra.1516

Jones, R.T., Robeson, M.S., Lauber, C.L., Hamady, M., Knight, R. and Fierer, N.
2009. A comprehensive survey of soil acidobacterial diversity using
pyrosequencing and clone library analyses. *The ISME journal,* 3, 442-453.

Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A. and Janssen, P.H.
2003. Laboratory cultivation of widespread and previously uncultured soil
bacteria. *Applied and Environmental Microbiology,* 69, 7210-7215.

Juteau, P., Rho, D., Larocque, R. and Leduy, A. 1999. Analysis of the relative abundance of different types of bacteria capable of toluene degradation in a compost biofilter. *Applied microbiology and biotechnology,* 52, 863-868.

Kim, S.Y., Yoo, K.S., Kim, J.E., Kim, J.S., Jung, J.Y., Jin, Q., Eom, H.J. and Han, N.S. 2010. Diversity analysis of lactic acid bacteria in Korean rice wines by culture-independent method using PCR-denaturing gradient gel electrophoresis. *Food Science and Biotechnology,* 19, 749-755.

Kruskal, W.H. and Wallis, W.A. 1952. Use of ranks in one-criterion variance analysis. *Journal of the American statistical Association***,** 47, 583-621.

Legendre, P. and Gallagher, E.D. 2001. Ecologically meaningful transformations for ordination of species data. *Oecologia,* 129, 271-280.

Lemly, A.D. 1982. Modification of benthic insect communities in polluted streams: combined effects of sedimentation and nutrient enrichment. *Hydrobiologia,* 87, 229-245.

Lockaby, B.G., Governo, R., Schilling, E., Cavalcanti, G. and Hartsfield, C. 2005. Effects of Sedimentation on Soil Nutrient Dynamics in Riparian Forests. *Journal of Environmental Quality,* 34, 390-396.

Macarthur, R.H. and Wilson, E.O. 1967. *The theory of island biogeography,* Princeton Univ Pr.

Maloney, K., Mulholland, P. and Feminella, J. 2005. Influence of Catchment-Scale Military Land Use on Stream Physical and Organic Matter Variables in Small Southeastern Plains Catchments USA. *Environmental Management,* 35, 677-691.

Maloney, K.O. and Feminella, J.W. 2006. Evaluation of single- and multi-metric benthic macroinvertebrate indicators of catchment disturbance over time at the Fort Benning Military Installation, Georgia, USA. *Ecological Indicators,* 6, 469-484.

Maloney, K.O., Munguia, P. and Mitchell, R.M. 2011. Anthropogenic disturbance and landscape patterns affect diversity patterns of aquatic benthic macroinvertebrates. *Journal of the North American Benthological Society,* 30, 284-295.

Maloney, K.O. and Weller, D.E. 2011. Anthropogenic disturbance and streams: land use and land-use change affect stream ecosystems via multiple pathways. *Freshwater biology,* 56, 611-626.

Merritt, R.W. and Cummins, K.W. 1996. *An introduction to the aquatic insects of North America,* Kendall Hunt.

Mikkonen, A., Lappi, K., Wallenius, K., Lindström, K. and Suominen, L. 2011. Ecological inference on bacterial succession using curve based community fingerprint data analysis, demonstrated with rhizoremediation experiment. *FEMS Microbiology Ecology*. DOI: 10.1111/j.1574-6941.2011.01187.x

Naiman, R.J., Decamps, H. and Mcclain, M.E. 2005. *Riparia: ecology, conservation, and management of streamside communities,* Academic Press.

Navel, S., Mermillod-Blondin, F., Montuelle, B., Chauvet, E., Simon, L. and Marmonier, P. 2010. Water-Sediment Exchanges Control Microbial

Processes Associated with Leaf Litter Degradation in the Hyporheic Zone: a Microcosm Study. *Microbial Ecology*, 61, 968-979.

Newcombe, C.P. and Macdonald, D.D. 1991. Effects of Suspended Sediments on Aquatic Ecosystems. *North American Journal of Fisheries Management,* 11, 72-82.

Nossa, C.W., Oberdorf, W.E., Yang, L., Aas, J.A., Paster, B.J., Desantis, T.Z., Brodie, E.L., Malamud, D., Poles, M.A. and Pei, Z. 2008. Design of 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome. *World Journal of Gastroenterology: WJG,* 16, 4135-4144.

Petersen, R.C. and Cummins, K.W. 1974. Leaf processing in a woodland stream. *Freshwater biology,* 4, 343-368.

Rappe, M.S. and Giovannoni, S.J. 2003. The uncultured microbial majority. *Annual Reviews in Microbiology,* 57, 369-394.

Reeder, J. and Knight, R. 2010. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nature methods,* 7, 668-669.

Reice, S.R. 1974. Environmental patchiness and the breakdown of leaf litter in a woodland stream. *Ecology*, 55, 1271-1282.

Resh, V.H., Brown, A.V., Covich, A.P., Gurtz, M.E., Li, H.W., Minshall, G.W., Reice, S.R., Sheldon, A.L., Wallace, J.B. and Wissmar, R.C. 1988. The role of disturbance in stream ecology. *Journal of the North American Benthological Society,* 7, 433-455.

Ryan, P.A. 1991. Environmental effects of sediment on New Zealand streams: a

    review. *New Zealand Journal of Marine and Freshwater Research,* 25,

    207-221.

Sait, M., Davis, K.E.R. and Janssen, P.H. 2006. Effect of pH on isolation and

    distribution of members of subdivision 1 of the phylum Acidobacteria

    occurring in soil. *Applied and Environmental Microbiology,* 72, 1852-1857.

Saito, A., Ikeda, S., Ezura, H. and Minamisawa, K. 2007. Microbial community

    analysis of the phytosphere using culture-independent methodologies.

    Microbes and Environments, 22, 93-105.

Santmire, J.A. and Leff, L.G. 2007. The influence of stream sediment particle

    size on bacterial abundance and community composition. *Aquatic*

    Ecology, 41, 153-160.

Sponseller, R.A. and Benfield, E.F. 2001. Influences of land use on leaf

    breakdown in southern Appalachian headwater streams: a multiple-scale

    analysis. *Journal of the North American Benthological Society,* 20, 44-59.

Suberkropp, K. and Klug, M.J. 1976. Fungi and bacteria associated with leaves

    during processing in a woodland stream. *Ecology,* 57, 707-719.

Suzuki, M.T. and Giovannoni, S.J. 1996. Bias caused by template annealing in

    the amplification of mixtures of 16S rRNA genes by PCR. *Applied and*

    Environmental Microbiology, 62, 625-630.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. 2007. Naive Bayesian

    classifier for rapid assignment of rRNA sequences into the new bacterial

    taxonomy. *Applied and Environmental Microbiology,* 73, 5261-5267.

Webster, J.R. and Benfield, E.F. 1986. Vascular plant breakdown in freshwater
ecosystems. *Annual Review of Ecology and Systematics,* 17, 567-594.

Webster, J.R. and Waide, J.B. 1982. Effects of forest clearcutting on leaf
breakdown in a southern Appalachian stream. *Freshwater biology,* 12,
331-344.

Witkamp, M. 1963. Microbial populations of leaf litter in relation to environmental
conditions and decomposition. *Ecology,* 44, 370-377.

Witkamp, M. 1966. Decomposition of leaf litter in relation to environment,
microflora, and microbial respiration. *Ecology*, 194-201.

Zar, J.H. 1999. *Biostatistical analysis,* Prentice hall Upper Saddle River, NJ.

Table 5.1. Study streams at Fort Benning Military Installation (FBMI) identified by their military compartment, UTM coordinates, predominant land use practices, and disturbance index value (proportion of watershed as bare ground and road cover). Disturbance intensity values from Maloney *et al.* (2005).

| Stream	Military compartment	Abbreviation	UTM	Military land use	Stream order	Disturbance intensity
Lois Creek	K13	LC	0715377N, 3597908E	Infantry/ ranger	2	3.67
Kings Mill Creek Tributary	K11	KM	0720701N, 3600036E	Infantry/ ranger	2	4.63
Sally Branch Tributary	F3	SB1	0716349N, 3585850E	Heavy machinery	3	8.38
Bonham Tributary	D12	BC	0710893N, 3588286E	Infantry/ ranger/ impact	2	10.46
Sally Branch Tributary	F1	SB2	0716005N, 3584889E	Heavy machinery	1	13.56
Sally Branch Tributary	D6	SB3	0714935N, 3585249E	Heavy machinery	2	14.66

Table 5.2. MID barcodes used to tag each PCR product during pyrosequencing of bacterial assemblage.

Primer Name	MID Barcode
MID 29	ATCAGACACG
MID 30	TGATACGTCT
MID 31	CGTCTAGTAC
MID 32	TACTCTCGTG
MID 33	ACTGTACAGT
MID 34	CAGTAGACGT
MID 35	TAGTGTAGAT
MID 36	ATATCGCGAG
MID 37	TACTGAGCTA
MID 38	TCTACGTAGC
MID 39	TAGAGACGAG
MID 40	AGACTATACT

Table 5.3. Mean ± 1SE current velocity, depth, and temperature for each site and sediment disturbance treatment.

Variable	Low Sediment			High Sediment		
Site	KM	LC	SB1	BC	SB2	SB3
Current velocity (m/s)	0.10 ± 0.01	0.06 ± 0.01	0.18 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	0.07 ± 0.01
Depth (m)	0.12 ± 0.01	0.15 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.08 ± 0.01	0.10 ± 0.01
pH*	4.33	4.04	5.13	3.92	4.22	5.31
Temperature (°C)	10.03 ± 0.31	10.11 ± 0.30	10.09 ± 0.33	10.35 ± 0.29	10.54 ± 0.28	10.18 ± 0.27

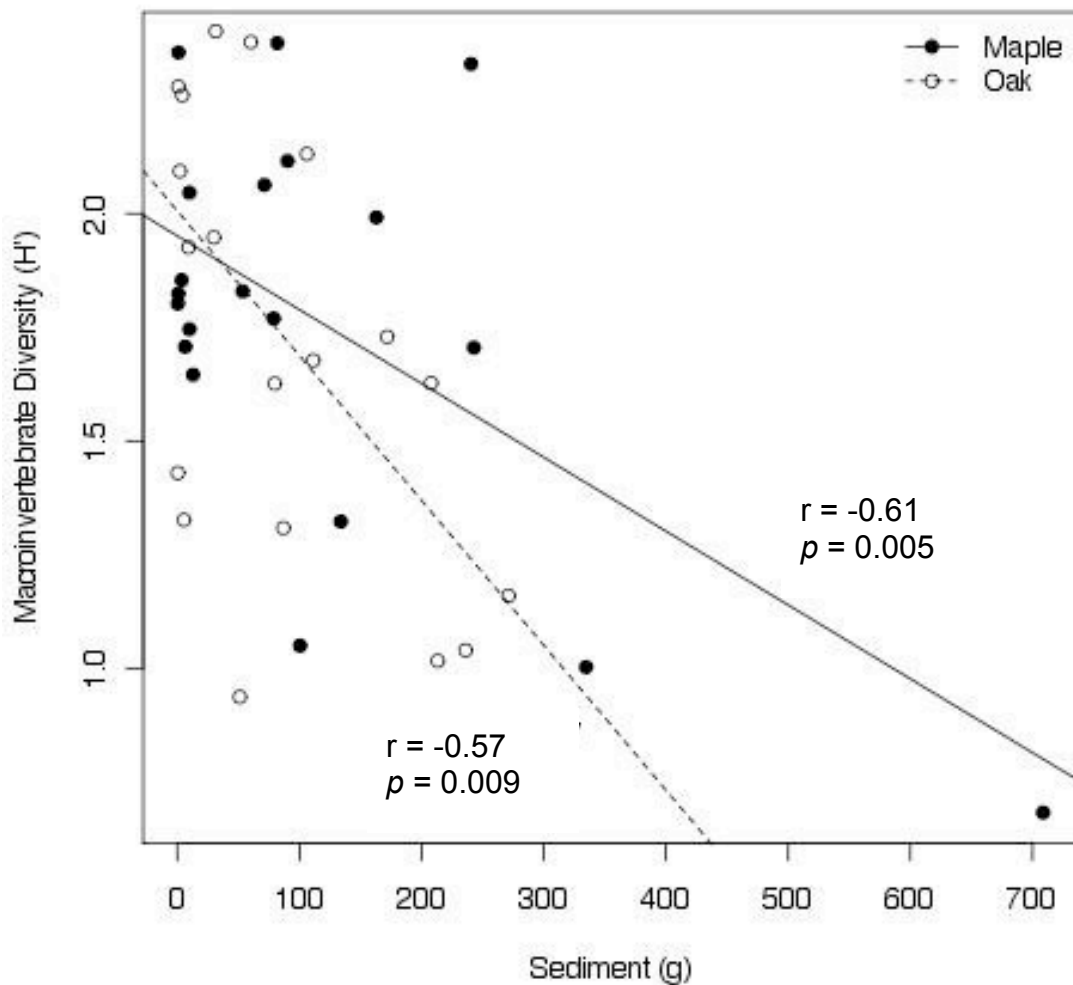* Duplicate values were not recorded for streamwater pH.
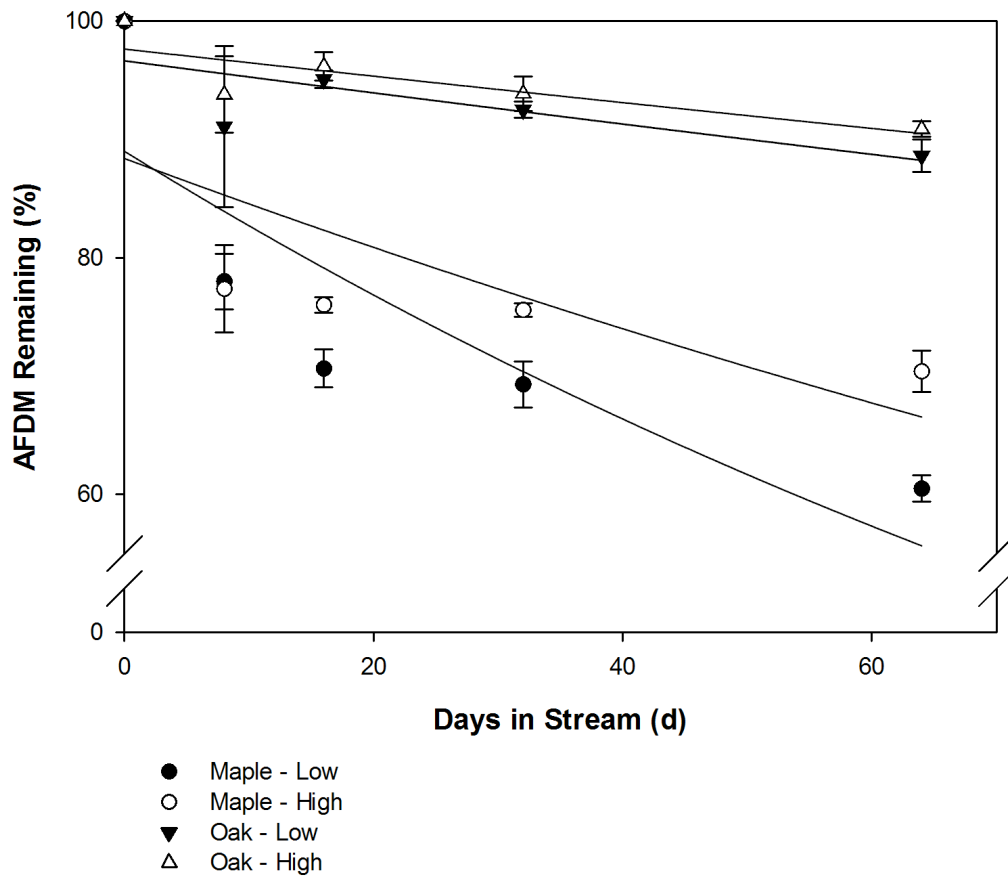
Figure 5.1. Leaf litter ash-free dry mass (AFDM) remaining (%) over time from low (solid) and high (hollow) sediment disturbance sites for both maple (circles) and oak (triangles). Plotted points are means (± 1SE).
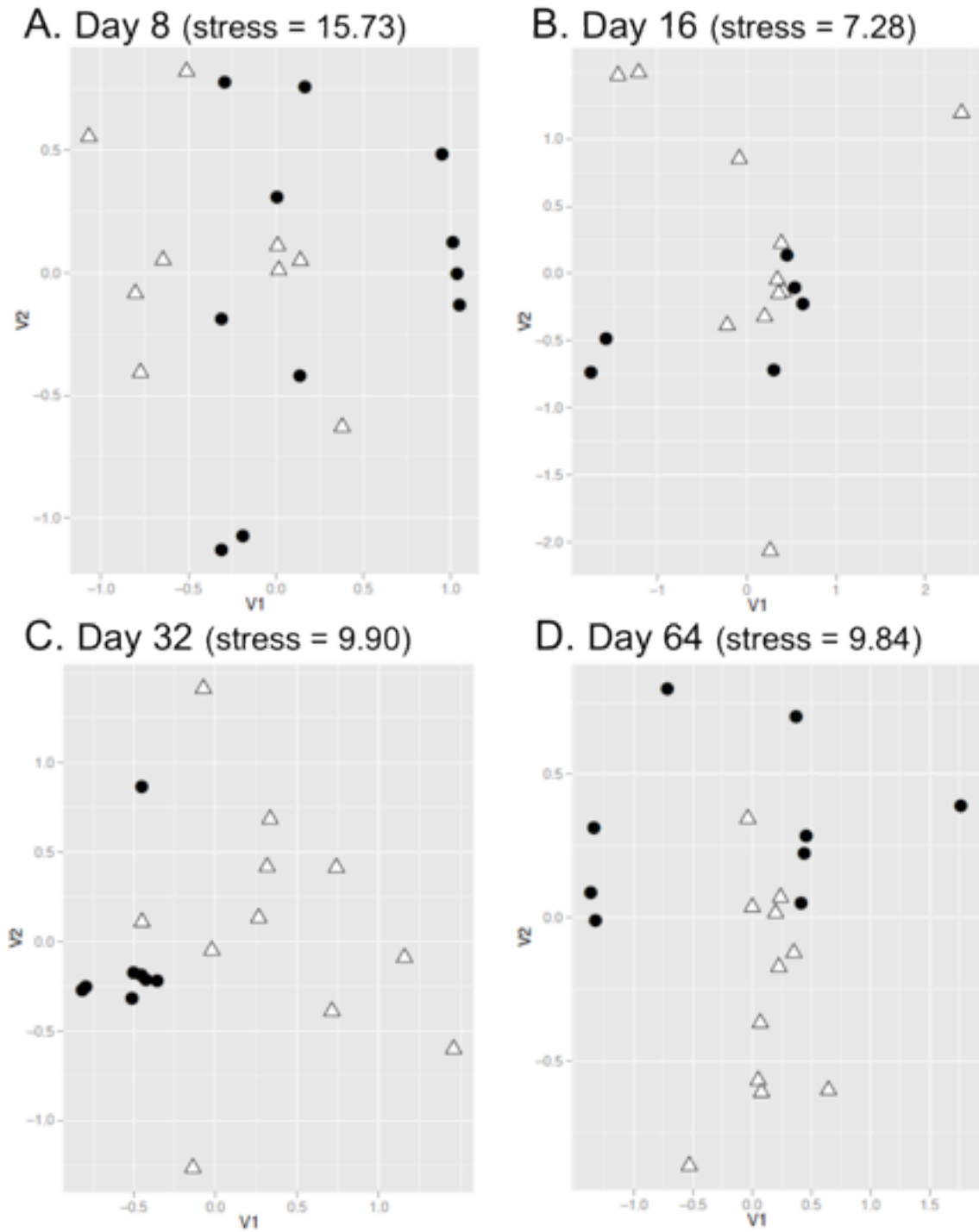
Table 5.4. Macroinvertebrate metrics (mean ± 1SE) for each leaf species and disturbance treatment combination during our 64-day incubation. Bolded values represent significant differences ($P < 0.05$) between low- and high-sediment disturbance sites.

	Maple			Oak		
	Low	High	p	Low	High	p
Macroinvertebrate abundance	26.56 ± 11.68	17.11 ± 10.81	**0.024**	23.46 ± 11.42	10.78 ± 7.63	**0.006**
Shredder abundance	7.14 ± 4.85	5.43 ± 3.85	0.211	8.26 ± 6.00	2.73 ± 1.89	**0.012**
Richness	7.10 ± 2.08	4.56 ± 1.80	**0.014**	6.42 ± 1.84	3.58 ± 1.50	**0.004**
Shannon's diversity (H')	1.40 ± 0.24	0.98 ± 0.24	**0.01**	1.26 ± 0.23	0.80 ± 0.26	**0.004**
Biomass (mg)	10.74 ± 3.84	3.05 ± 2.32	**0.001**	5.64 ± 3.14	1.62 ± 1.28	**0.002**
Macroinvertebrate density (ind./g AFDM)	14.04 ± 6.90	7.90 ± 5.21	**0.015**	7.48 ± 3.87	3.45 ± 2.45	**0.009**
Shredder density (ind./g AFDM)	3.93 ± 2.81	2.54 ± 1.88	0.172	2.70 ± 1.99	0.86 ± 0.61	**0.015**
% Chironomidae	64.14 ± 2.04	64.47 ± 3.98	0.876	68.32 ± 4.05	65.66 ± 4.92	0.914
% EPT	15.82 ± 1.54	13.50 ± 3.66	0.999	12.05 ± 5.21	15.50 ± 3.88	0.928

Figure 5.2. Diversity of macroinvertebrates (*H'*) and associated sediment (grams) in maple (solid circles) and oak (hollow circles) leaf packs. Spearman's rank correlations were used to describe the macroinvertebrate diversity to sediment relationships. Trend lines shown indicate significant relationships (*p*<0.05).

Figure 5.3. Macroinvertebrate taxon richness (S) and associated sediment (grams) in maple (solid circles) and oak (hollow circles) leaf packs. Spearman's rank correlations were used to describe the macroinvertebrate taxa richness to sediment relationships. Trend lines shown indicate significant relationships ($p<0.05$).
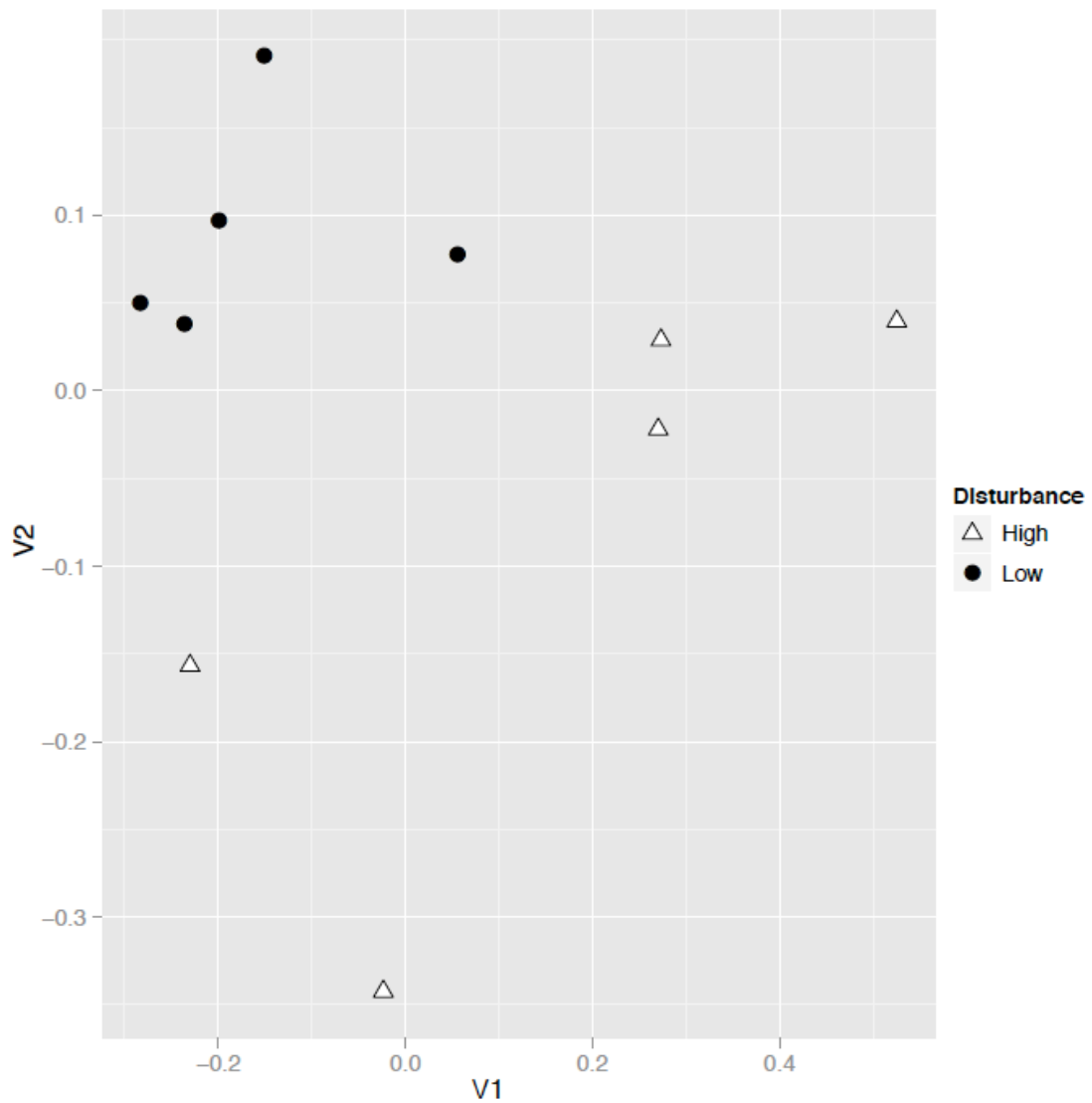
Figure 5.4. Nonmetric multi-dimensional scaling (NMDS) plots based on Bray-Curtis similarities between maple leaf litter samples from low- (•) and high- (Δ) sediment disturbance sites for days 8, 16, 32, and 64.
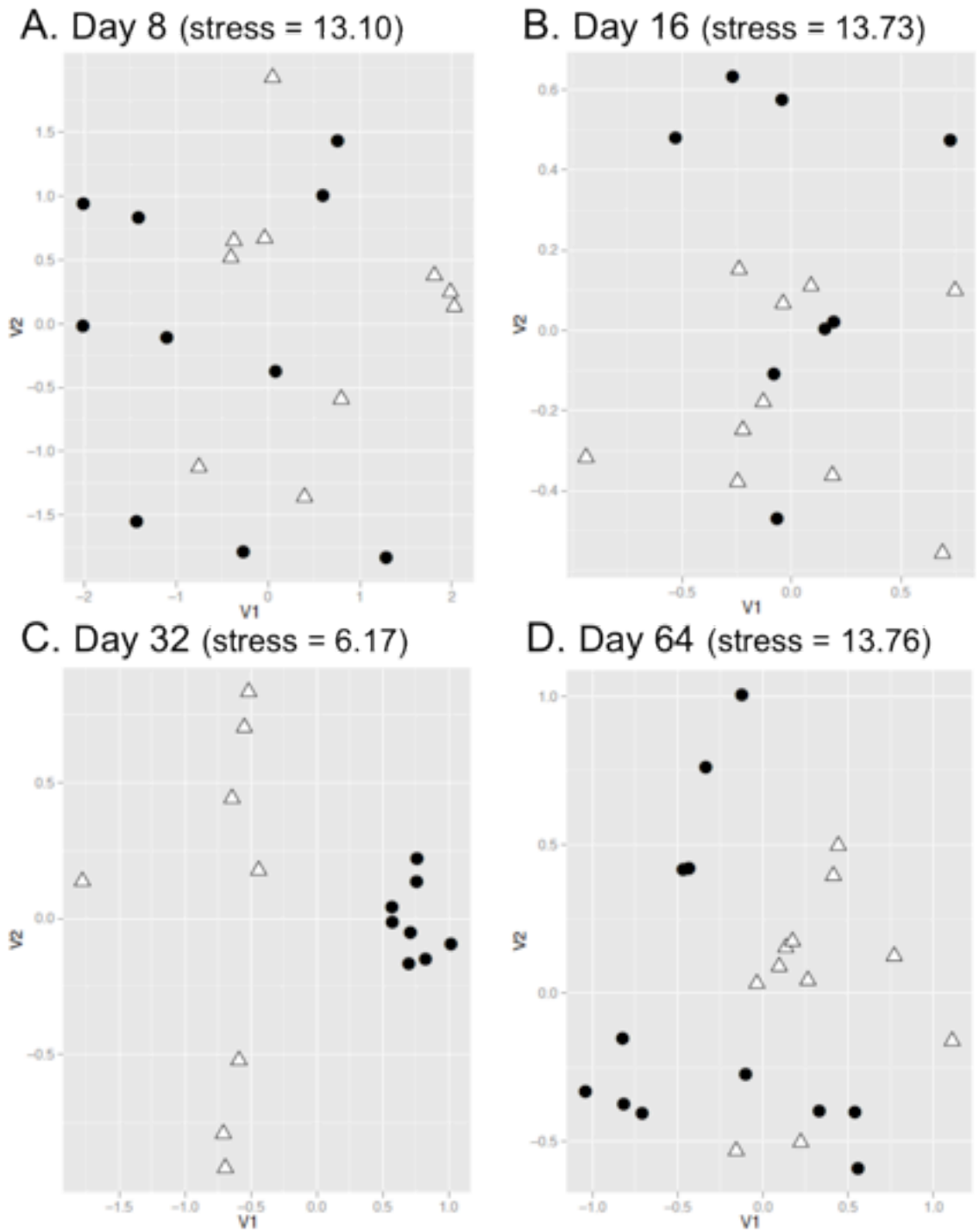
Figure 5.5. Nonmetric multi-dimensional scaling (NMDS) plots based on Bray-Curtis similarities between oak leaf litter samples from low- (•) and high- (Δ) sediment disturbance sites for days 8, 16, 32, and 64.

Table 5.5. Mean ± 1SE diversity, richness, and OTU estimates for each time and sediment disturbance treatment sampled during pyrosequencing.

Index[a]	Day 32		Day 64	
	Low	**High**	**Low**	**High**
Shannon (H')	5.8 ± 0.6	5.7 ± 0.1	5.6 ± 0.5	6.2 ± 0.4
Chao1	182.1 ± 62.5	156.8 ± 12.3	169.4 ± 27.7	255.9 ± 2.3
OTUs	81.5 ± 18.7	76.2 ± 0.9	77 ± 15.4	100.9 ± 13.9

[a] Index values are normalized based on treatment with smallest number of sequences (Day 64, High).

170

Figure 5.6. Nonmetric multi-dimensional scaling (NMDS) plots derived from

Bray-Curtis dissimilarity values between maple leaf litter samples from

pyrosequenced low- (•) and high- (Δ) sediment disturbance sites for days 32 and

64. Stress level = 0.081.
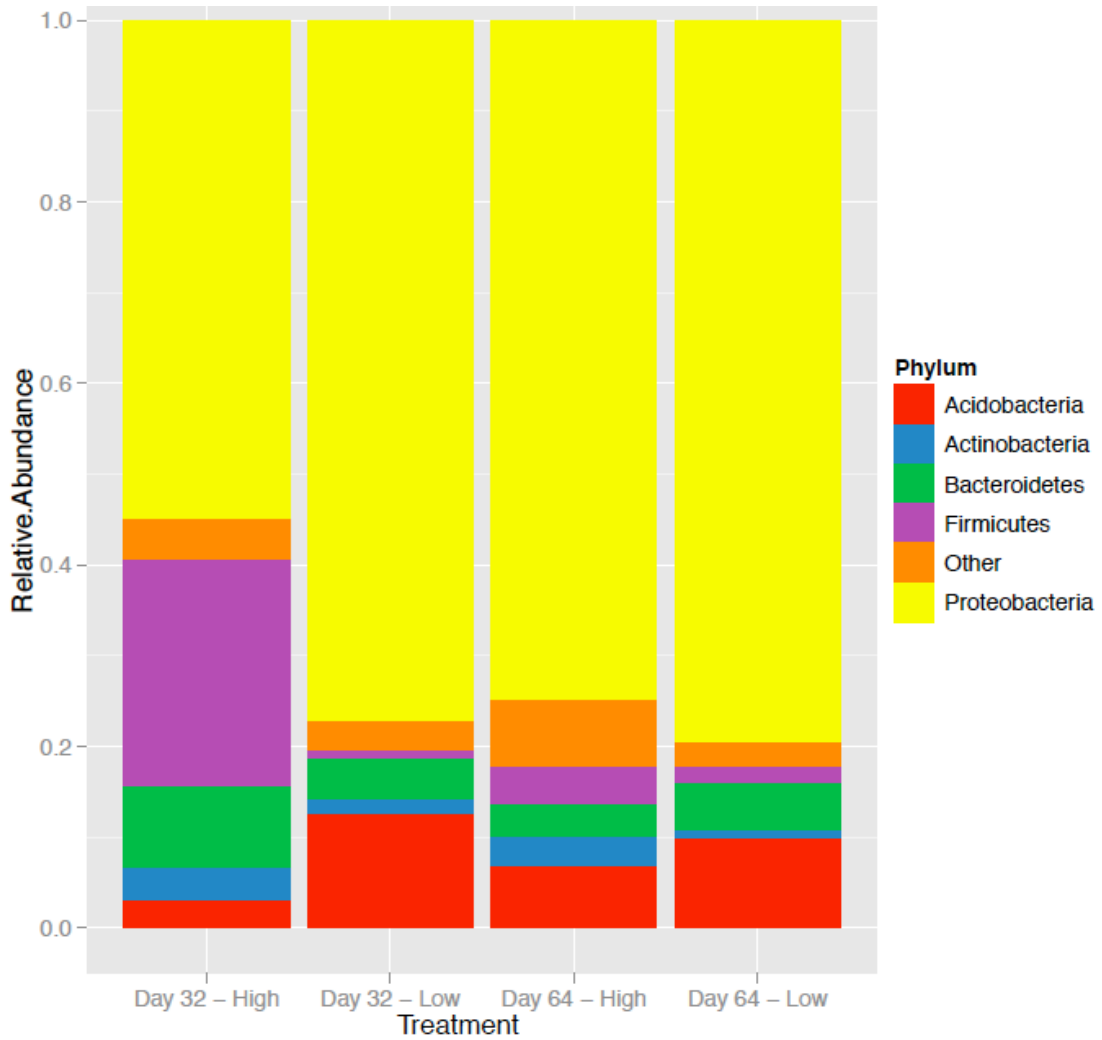
Figure 5.7. Comparison of relative abundance of the most common bacterial phyla found in pyrosequenced maple leaf pack samples grouped by days in stream and sediment disturbance (high vs. low).
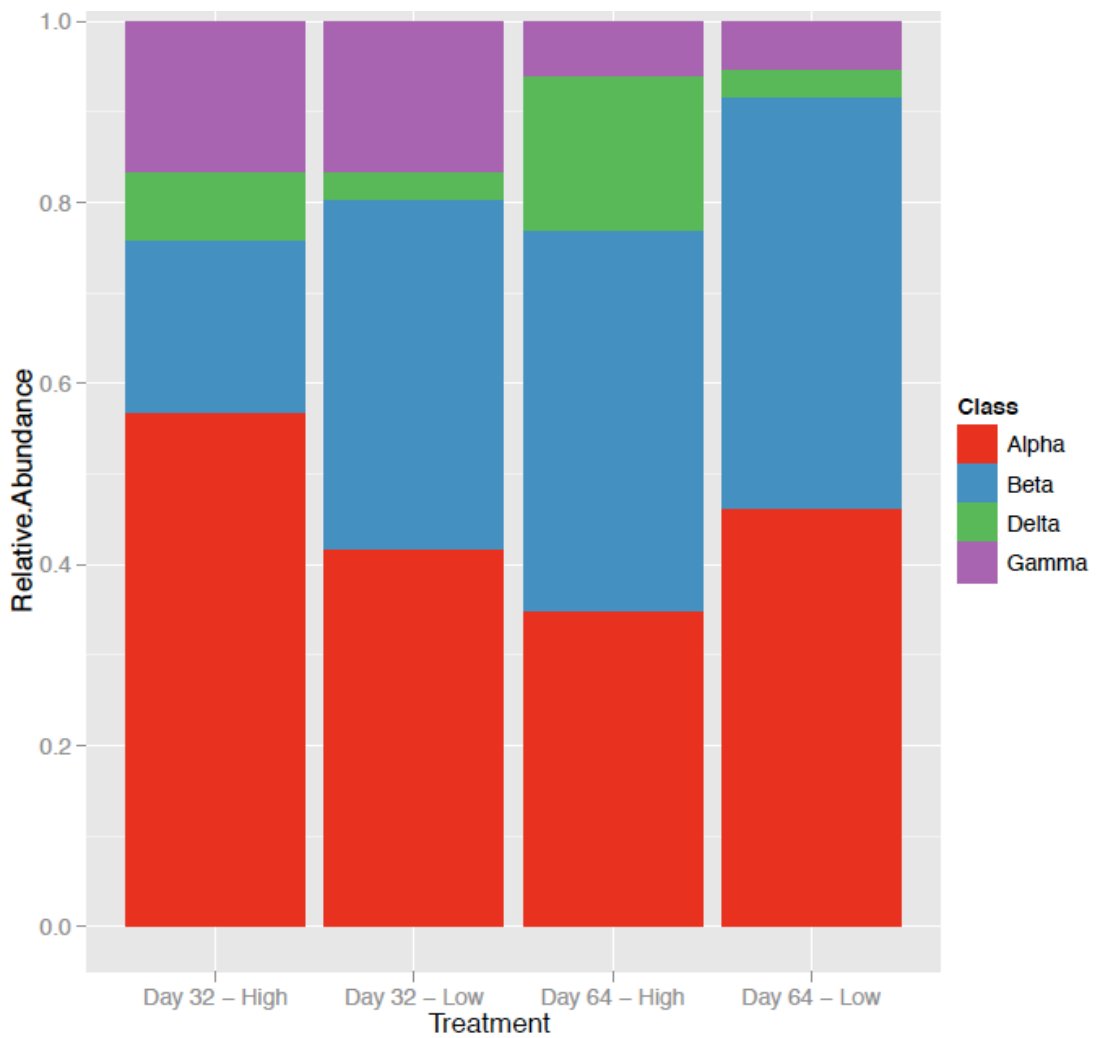
Figure 5.8. Comparison of relative abundance of Proteobacteria classes found in pyrosequenced maple leaf pack samples grouped by days in stream and sediment disturbance (high vs. low).
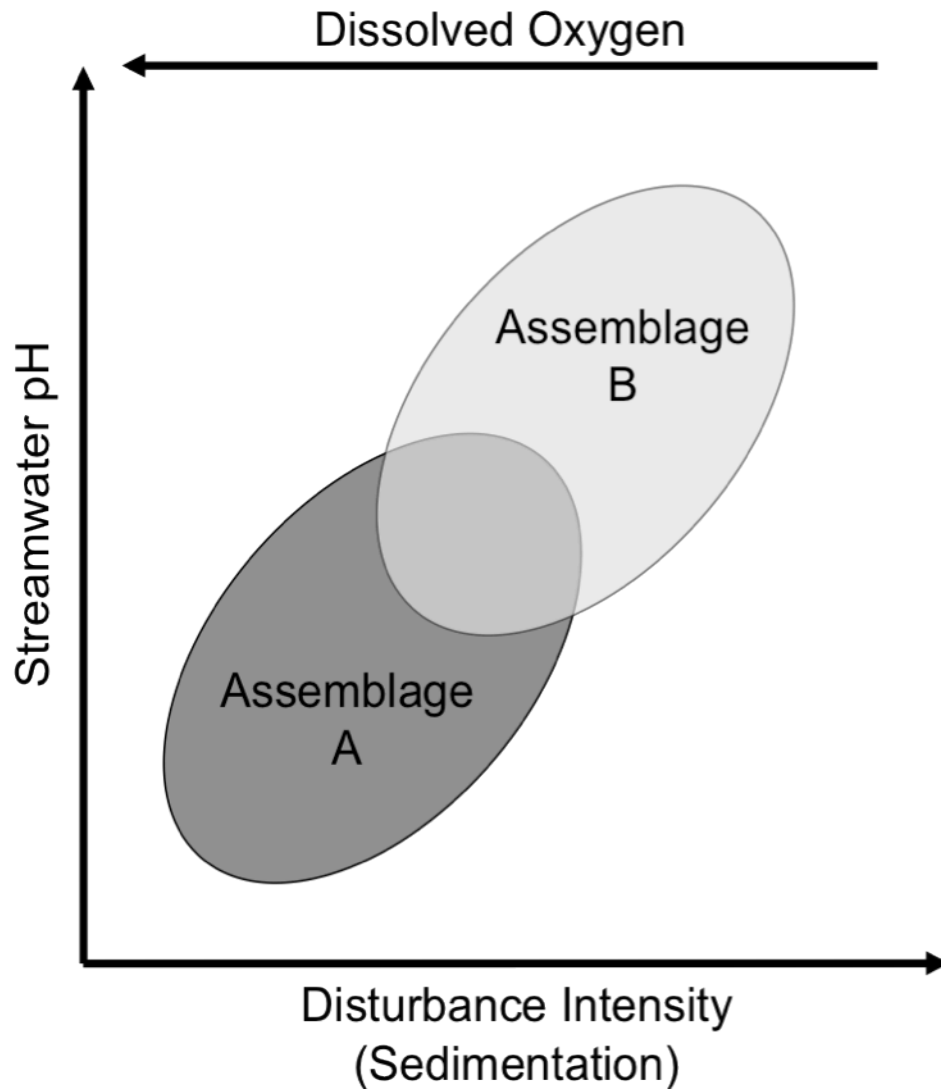
Figure 5.9. Conceptual model illustrating the predicted shift in leaf pack bacterial assemblage composition under increased sedimentation and catchment disturbance intensity (see Maloney *et al*., 2005 for disturbance intensity estimation) toward a bacterial assemblage dominated by taxa capable of surviving harsher environmental conditions (e.g. increased streamwater pH and decreased dissolved oxygen).