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Microbial Communities Colonizing Leaves during Early Decomposition Stages

Stephanie Harper

MICROBIAL COMMUNITIES COLONIZING LEAVES DURING EARLY
DECOMPOSITION STAGES

by

STEPHANIE HARPER

Under the Direction of Checo Colon-Gaud

ABSTRACT

Microbial communities associated with decaying leaves play an important role in the

cycling of nutrients in stream ecosystems. In headwater streams that are deemed as

heterotrophic, bacteria and fungi are main drivers of organic matter decomposition and thus

partly responsible for facilitating the cycling of nutrients from leaves that fall into the stream.

The main objective of this study was to compare microbial community composition between

different leaf types during breakdown in stream ecosystems. To achieve this objective, I used a

combination of field and laboratory trials. Field experiments were performed at the Luquillo

Experimental Forest using *Dacryodes excelsa* and *Cecropia schreberiana* leaves in June 2012

and at the Coweeta Long Term Ecological Research site using *Acer rubrum* and *Quercus prinus*

leaves in June 2013. Laboratory trials using the same leaf types were set up in experimental

chambers to model the systems found in these two regions. Decay rates and microbial

communities were analyzed for individual leaf types during field and laboratory experiments.

Although decay rates between leaf types in the field experiment did not differ, results from the

laboratory trials suggest that *A. rubrum* has higher decay rates and thus decomposes faster than

the other leaf types examined. Results also suggest that individual taxa colonizing leaves

differed between leaf types but microbial community richness and Shannon's diversity did not

differ. These results suggest that different leaf types may harvest unique microbial communities

responsible for facilitating the decay process even if these leaves are exposed to similar

environmental conditions (i.e., decaying in the same stream or region).

INDEX WORDS: Leaf Breakdown, Bacteria, Fungi, Macroinvertebrates, Stream Ecosystems, T-RFLP, Microbial Diversity

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by

STEPHANIE HARPER

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STEPHANIE HARPER

Major Professor: Checo Colon-Gaud

Committee:　　　John Scott Harrison

　　　　　　　　Tiehang Wu

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DIEDICATION

I dedicate this body of research to my family, who have taught me that hard work and determination are the keys success. I appreciate all of their love and support over the years that have made this experience possible.

I would also like to dedicate this to the scientists and teachers in the past who have challenged me and accepted nothing but the best from me. Their guidance and knowledge have inspired me to be curious about the natural world and I hope that someday I will be able to pay that inspiration forward.

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CHAPTER 1

INTRODUCTION

Leaf breakdown in forested headwater streams is a major ecosystem process because it fuels the stream with energy (Vannote et al. 1980). Once entering the system, leaves go through three decomposition stages: leaching, conditioning, and fragmentation (Petersen and Cummins 1974). Consumers within stream ecosystems responsible for leaf breakdown include microbes, such as bacteria and fungi, as well as the invertebrate consumers known as shredders (Gessner et al. 1999), Wallace and Webster 2006,). When microbes colonize leaves, they facilitate leaf conditioning and initiate biological breakdown of leaf constituents. In doing so, nutrients become available to higher trophic levels (Barlocher 1985, Graca et al. 1993, Wong et al. 1998).

During leaf breakdown, bacteria and fungi are responsible in the processing of leaf constituents (i.e., lignin, tannins, and phenolics). Their ability to breakdown complex compounds allows microbes to condition and increase leaf quality for invertebrate consumers (Petersen and Cummins 1974, Cummins et al. 1989, Abelho 1993, Graca 2001). Different leaf types vary in leaf chemistry and therefore vary in their overall quality to consumers. Leaves with high concentrations of leaf constituents are considered to be low quality resources and decrease decay rates and thus exhibiting slower decomposition rates. Initial concentration of lignin in leaves has been found to be negatively correlated to decay rates (Campbell and Fuchshuber 1995, Ardon et al. 2009). Subsequently, microbial communities on leaf substrates and the succession of those microbes over time may be explained by leaf chemistry. As microbes begin to degrade the compounds in leaves, the quality of the leaf substrate changes and initially increases until only refractory materials remains (Webster and Benfield 1986, Abelho 2001, Kominoski et al. 2009). Changes in leaf quality throughout the process of breakdown could cause microbes to follow a predictable pattern in community composition over time. While

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bacteria and fungi are both successful at breaking down leaves, they break down different types of leaf constituents more efficiently and in turn influence microbial community structure (Wymore et al. 2013). Fungi have been shown to exhibit higher enzymatic activities related to the degradation of plant polymers (i.e., lignin, cellulose, and hemicellulose). In contrast, bacteria have been shown to exhibit higher enzymatic activities related to the acquisition of N and P. This suggests that bacteria and fungi process leaf constituents differently in order to assimilate necessary nutrients (Romani et al. 2006).

In addition to leaf constituents, available nutrients as well as the nutritional composition of leaf substrates may influence microbial communities. In a 5 year study on a N and P enriched stream, fungal biomass, fungal production, and fungal sporulation were higher after nutrient enrichment compared to prior to nutrient additions, as well as higher than at a reference stream (Superkropp et al. 2010). A closer look determined that fungal biomass responded more when both N and P were added (instead of when they were added separately) (Rosemond et al. 2008). It has been suggested that fungi are N and P limited and could explain the importance of these nutrients for fungal biomass, production, and sporulation (Howarth and Fisher 1976). In contrast, bacteria has shown little response to P addition, reinforcing the idea that bacteria are C limited (Chróst and Ria 1993, Suberkropp et al. 2010).

Microbial biomass throughout the process of decomposition suggests that microbes follow a predictable pattern throughout leaf breakdown. Fungi has been found to be more abundant initially, whereas bacteria increase in abundance slowly throughout breakdown (Suberkropp and Klug 1976, Wright and Covich 2005, Duarte et al. 2010). This was originally attributed to the mechanical process and enzymatic capabilities that allow bacteria and fungi to breakdown compounds (Suberkropp and Klug 1976, Suberkropp et al. 2010). However, with

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recent improvements to microbial methodologies (enzyme activity and molecular techniques), bacteria has been found to be abundant in the early stages of breakdown as well (Romani et al. 2006, Duarte 2010, Wymore et al. 2013). Therefore, bacteria may be more important in the early stages of leaf breakdown than previously believed. With more fine scale techniques we can better assess entire microbial communities and the succession of these communities throughout the entire process of leaf breakdown (Barlocher 2007).

Another factor that could determine patterns in community composition of microbes throughout the different stages of leaf breakdown is the interaction between bacteria and fungi. When only fungi or bacteria are present on leaf substrates, competition between different species for space and resources appear to have minimal effects on community composition (Meidute et al. 2008). This suggests that when only one type of microbes (bacteria or fungi) are present, they may be able to partition resources better than when both types (bacteria and fungi) are present. However, when both bacteria and fungi are grown together on leaf substrates, they differ in their abundance. When fungi are grown in the presence of bacteria, they are less abundant and do not grow as large as when grown alone. In contrast, when bacteria are grown in the presence of fungi, they are more abundant than when grown alone (Wohl and McArthur 2001, Mille-Lindblom and Tavernik 2003). Fungi may be crucial to the success of bacteria on leaf substrates because fungi facilitate bacterial colonization by exposing different leaf parts. For example, due to their feeding strategies, the presence of fungi may allow bacteria to colonize newly exposed sections under the leaf cuticle that would not be available if only bacteria were present. (Suberkropp and Klug 1976, Bergfur and Friberg 2012).

Differences in microbial community composition can also play an important role in the rate of leaf breakdown (Pascoal et al. 2010, Fernandes et al. 2011, and Perez et al. 2012). When

investigating the effects of aquatic hyphomycete species identity and diversity on leaf breakdown rates, species diversity did not cause a significant different in leaf breakdown rates. Instead, aquatic hyphomycete species identity did influence the rate at which leaves broke down (Duarte et al. 2006). This suggests that different species may be important drivers but the overall community colonizing leaf materials may demonstrate redundancy where there are many taxa performing the same or similar functions within the community. In addition, fungal communities with low diversity (4 species) and high diversity (8 species) did not have significantly different decay rates (Geraldes et al 2012). Therefore, community structure rather than community diversity may be more important when determining the influences that microbial communities have on decay rates.

Microbial colonizers are also known to facilitate consumption of leaf material by macroinvertebrates known as shredders. After microbes condition leaf substrates, shredders are responsible for further breaking down leaf litter by shredding the leaf material. (Petersen and Cummins 1974, Covich et al.1999). Biologically-mediated fragmentation changes leaf litter from coarse particular organic matter (CPOM) to fine particular organic matter (FPOM), which makes the resource available to other functional feeding groups such as filterers and collector-gatherers (Wallace and Webster 1996, Covich et al. 1999, Graca and Canhoto 2006). In addition, shredders feed directly on microbes and therefore, leaves are simply processed due to the proximity to microbes (Cummins et al. 1989, Arsuffi and Suberkropp 1984, Arsuffi and Suberkropp 1985, Arsuffi and Suberkropp 1989, Graca 2001). Arsuffi and Suberkropp (1984) used a leaf shredding caddisfly to assess consumer preference of leaves colonized by one of four fungal species. They found that the preference of the caddisfly shredder was dependent on the fungal species they were able to feed on and how long they were given to feed. Further, Arsuffi and Suberkropp

(1985) found that caddisfly (*Hesperophylax* and *Psychoglypha*) shredders were able to locate colonized areas when fed leaves inoculated with fungal patches. More recently Chung and Suberkropp (2009) found that invertebrate shredders had negative growth on sterile leaves that contained no microbes. Thus indicating that fungal biomass is a sufficient food source because it influences and stimulates growth of invertebrate shredders. Because invertebrate consumers have been found to assimilate microbes, they may influence the community structure of microbes on leaf substrates. If consumers selectively feed upon particular taxa of microbes, this interaction may cause a shift in community structure including a decrease in dominant fungal species or overall species diversity.

Global patterns in biodiversity have been observed for many taxa (Pianka 1966). There are numerous hypothesis that attempt to explain why biodiversity increases as latitude decreases (Willig et al. 2003). However, colonizing microbes and aquatic insects fail to exhibit the same latitudinal patterns in diversity as other taxa. Microbial communities have been shown to lack in latitudinal patterns but may be linked to other biogeography factors, such as pH (Fierer and Jackson 2006). In addition many tropical regions are believed to have a paucity of insect shredders, however, in these streams other macroconsumers (i.e., shrimps, crabs, snails) assume the role of leaf processing. In a headwater stream in Puerto Rico, decay rates where shrimp and insects were excluded individually from leaves were not significantly different. Failure to observe large changes in decay rates between invertebrate treatments suggests that a paucity of insect shredders may not be as drastic as once believed. Because of differences in aquatic insect communities, Irons et al. (1994) suggested that microbes could have a greater role in leaf breakdown in tropical streams compared to temperate streams.

In addition to the lack of patterns in diversity, there have been observed differences in decay rates between streams in temperate and tropical regions. Although leaves go through similar stages of decay in temperate and tropical headwater streams, tropical headwater streams exhibit higher breakdown rates (Irons et al. 1994, Abelho et al. 2005, Wright and Covich 2005, Goncalves et al. 2006). The obvious difference of abiotic factors, such as temperature, may be one explanation of observed difference in decay rates. However, temperature effects may not be the only driving factor for these differences. While temperature has been suggested to influence the rate of leaf breakdown (Geraldes et al. 2012), a study investigating the latitudinal gradient of breakdown rates found that temperature many not be solely driven by expected differences in water temperature (Irons et al. 1994). Additional studies have attempted to identify the driving factors of differences in leaf breakdown between temperate and tropical regions by investigating leaf quality (Ardon et al. 2009), temperature (Goncalves et al. 2006), microbial activity (Ferreriera et al. 2012), and invertebrate diversity (Boyero et al. 2012).

The main objective of this study was to investigate how bacterial and fungal communities associated with leaf substrates differ among different leaf types during decomposition in stream ecosystems. I measured community composition, abundance, richness, and diversity on four leaf types at four time intervals. I hypothesized that microbial community composition would differ between leaf types and regions. I also hypothesized that microbial communities would change over time and follow a predictable pattern of microbial succession. These studies were performed through a series of field and laboratory experiments.

CHAPTER 2

Microbial community composition during breakdown in a temperate and tropical headwater stream

INTRODUCTION

Organic matter processing is an important ecosystem process in small forested headwater streams that are deemed heterotrophic (Vannote et al. 1980). Organic matter (cycling) between streams in temperate and tropical regions has been a major research focus. Although streams in each region can be highly variable (Boulton et al. 2009, Boyero et al. 2009), major comparisons were initiated when Irons et al. (1994) suggested that temperature is not the driving factor between differences in leaf decay rates. Leaf chemistry has been used to determine differences between regions, however, contrary to expectations, results suggest that both regions contain leaf types with wide variation in their initial leaf chemistry (Campbell and Fuchshuber 1995). Further, Ardon et al. 2008 investigated the influence of secondary compounds on decay rates in a tropical stream. They found that secondary compounds (i.e., lignin, tannins) were leached immediately and decay rates were influenced more by the physical structural compounds (i.e., cellulose) rather than the secondary compounds.

In addition, many streams in tropical regions have been described as having a paucity of insect shredders (Boyero et al. 2009). This has been supported in select tropical streams in Kenya, Brazil, and Hong Kong (Dobson et al. 2002, Wantzen and Wagner 2006, Li and Dudgeon 2009). This may be linked to the life history of shredders and their evolution in forested headwater streams with low temperature (Jacobsen et al. 2008). However, in these regions it is possible that leaf litter processing is facilitated by macroconsumers (i.e., shrimp, crabs, snails), rather than shredding insects. Irons et al. (1994) also suggested that in tropical streams due to

high temperatures and lack of insect shredders, microbes could be a driving force in the processing of organic matter.

Microbial communities associated with decaying leaves play an important role in the cycling of nutrients in stream ecosystems (Petersen and Cummins 1974, Cummins et al. 1989, Wong et al. 1998). Bactria and fungi process leaves by breaking down leaf compounds and making them available to other biota within the stream (Barlocher 1985, Graca et al. 1993, Wong et al. 1998). Microbial activity and community composition has been studied to determine abiotic and biotic influences on the overall contribution to leaf litter decay rates in streams (Chamier 1987, Suberkropp and Chauvet 1995, Heiber and Gessner 2002, Duarte et al. 2008, Krauss et al. 2011).

In streams where insect shredders are less abundant, increased microbial activity could be driving breakdown. Thus understanding microbial community composition and patters in community structure may be an important step in linking these organisms to this essential ecosystem process. Therefore, my goals with this project were to compare microbial community structure and overall community diversity on decaying leaves in forested headwater streams from two different regions (North Carolina and Puerto Rico). I hypothesized that microbial communities would differ between regions and to a lesser extent between leaf types due to differences in environmental conditions and leaf chemistry which results in differential species pools in each region. I predict that differences in environmental conditions and leaf chemistry will result in different microbial communities in each region. I also hypothesized that differences in microbial community structure would be associated with different stages of litter decay resulting in specific fungal and bacterial species driving decomposition rates at differing

temporal scales. I predict that certain fungal and bacterial communities will drive breakdown rates.

MATERIALS AND METHODS

Study Site

Leaf packs containing single-species leaf materials were deployed at two Long Term Ecological Research (LTER) sites; the Coweeta Hydrologic Laboratroy (hereafter Coweeta) in North Carolina and the Luquillo Experimental Forest (hereafter Luquillo) in Puerto Rico. The major tributaries in Luquillo are Quebradas Sonadora, Prieta, Toronja, and Gatos. The forest is characterized by unstable steep terrain. In these areas, yagrumo or pumpwoods (*Cecropia schreberiana*) and Sierra palm (*Prestoea montana*) are among the dominant tree species. Along riparian zones, thick canopy cover is attributed to dominant tree species such as tabonuco (*Dacryodes excelsa*) also known as gommier and candlewood (Covich and McDowell 1996). In Luquillo, collection and deployment of leaf packs were completed during June 2012.

A network of small headwater streams drains Coweeta where Ball Creek and Shope Fork join together to form Coweeta Creek. The forest at Coweeta is classified as a deciduous forest where red maple (*Acer rubrum*) and chestnut oak (*Quercus prinus*) are among the dominant species (Webster et al. 1999). In Coweeta, collection and deployment of leaf packs were completed during June 2013.

Preparation and processing of leaf packs

Two leaf types were collected from the riparian zones at each LTER site post abscission (Temperate: *Acer rubrum* or *Quercus prinus* and Tropical: *Dacryodes excelsa* or *Cecropia*

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schreberiana). Approximately 5g of each single leaf type were weighed and placed in coarse mesh bags (~10 mm openings). Leaf packs were attached to rebar and placed in Quebrada Gatos at the Luquillo LTER on 10 June 2012 and in stream C54 at the Coweeta LTER on 10 June 2013. We deployed leaf packs over a distance of 1 river kilometer in a randomized block design. At each site, eight replicate leaf packs were removed from the stream at set intervals (7, 21, 35, 49 days). In four replicates, leaf matter was removed and immediately stored in glycerol for microbial community assessment (Harrop et al. 2009). These samples were sent to the laboratory at Georgia Southern University and stored at -20°C. The remaining four replicates were used to measure leaf decay rates. To do so, leaves were rinsed of any colonizing invertebrates after being removed from the stream and consequently dried at 60°C for 48 h and weighted to the nearest 0.0001g. Leaf decay rates were estimated using an exponential decay model where k (decay coefficient) is estimated as the rate of original mass loss from the following equation:

$$k = Ln(OMR\%/100)/t,$$

where OMR% = the percent orgiginal mass remaining, and t = time in days (Petersen and Cummins 1974, Benfield 2006).

Microbial Community Analysis

To assess microbial community structure, environmental DNA was extracted from leaf material taken from replicate leaf packs using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, California) following the manufacturers' protocol for maximal yield. Bacteria 16S rDNA was amplified from extracted DNA using primers 8F (5-6-FAM/AGAGTTTGATCCTGGCTCAG-3) and 907R1 (5- CCGTCAATTCCTTTGAGTTT-3). Each reaction had a total of 10 μl and included 1X Apex Taq Master Mix (1.5mM $MgCl_2$,

Genesee Scientific, San Diego, California), 1 µM of each primer, 1 µl of DNA and sterilized water was added to reach the final volume. The reactions were performed with an initial denaturation at 94 ºC (3 min), followed by 30 cycles of denaturation at 94 ºC for 30s, annealing at 55ºC for 30s and extension at 72 ºC for 30s, plus a final elongation at 72 ºC for 5 min. PCR products were confirmed by gel electrophoresis.

Fungal ITS regions were amplified from extracted DNA using primers ITS1-F (5-C TTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3). The forward primer ITS1-F was labeled with a green HEX fluorescence tag. Each reaction had a total of 10 µl and included 1X Apex Taq Master Mix (1.5mM MgCl2, Genesee Scientific, San Diego, California), 1 µM of each primer, 1 µl of DNA and sterilized water was added to reach the final volume. The reactions were performed with an initial denaturation at 94 ºC (3 min), followed by 35 cycles of denaturation at 94 ºC for 60s, annealing at 55 ºC for 60s and extension at 72 ºC for 60s, plus a final elongation at 72 ºC for 10 min. PCR products were confirmed by gel electrophoresis.

PCR products of bacterial16S rDNA were digested with HaeIII and MspI and fungal ITS rDNA were digested with HaeIII and RsaI respectively. Bacterial digests included 1 X RE buffer, 0.25 U/µl of each restriction enzyme, 2 ul of PCR product, and sterilized water was added to a final volume of 10 ul. Fungal digests included 1 X RE buffer, 0.25 U/µl of each restriction enzyme, 2 µl of PCR product, and sterilized water was added to a final volume of 10 µl. PCR products were digested at 37 ºC for 6 h.

Restricted PCR products were loaded on an automated capillary electrophoresis sequencer (Applied Biosystems 3500 Genetic Analyzer, Foster City, California) to detect terminal restriction fragment length polymorphisms (T-RFLP). One µl digested PCR product

was mixed with 10 μl of HiDi Formamide and 0.5 μl of size standard Liz600 (Applied Biosystems, Foster City, California). Raw T-RFLP data was detected using GeneMapper ver. 3.7. Terminal Restriction Fragment (TRF) peaks were identified from individual T-RFLP profiles and relative abundance was calculated using peak area. Relative abundance for each sample was arranged and aligned by base pairs (±0.5) using TREEFLAP (Walsh, C. Monash Univ, http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls). Peaks comprising of <1% of total area along with bacterial peaks <100 bp and fungal peaks <50 bp were excluded from the analysis. Bacterial peaks <100 bp were excluded to avoid possible contamination that confounded samples in laboratory trails (Chapter 3).

Statistical Analyses

A non-parametric Kruskall Wallis test was used to determine the effects of leaf type on decay rates using JMP statistical package (v. 10.0 SAS Institute Inc. 2010). Microbial community profiles were analyzed using cluster analysis, non-metric multidimensional scaling (nMDS) and Analysis of Similarities (ANOSIM). Dissimilar communities were further analyzed with Similarity Percentages (SIMPER) to determine which TRFs were driving community differences. All multivariate analyses, including diversity indices, Bray-Curtis similarity, cluster analysis, non-metric multidimensional scaling (nMDS), and Analysis of Similarity (ANOSIM) were conducted using the PRIMER-E v.6 statistical software (Clarke and Gorley 2006). In addition, to test my hypothesis that microbial diversity is related to decay rates, community diversity indices (i.e., Evenness, Shannon's Diversity, Simpson's Diversity) were compared.

RESULTS

Leaf Breakdown

After the 49-day leaf pack experiment, mean percent leaf mass remaining ranged from 43.8% to 62.2% with *D. excelsa* having the lowest percent mass remaining and *Q. prinus* having the highest percent mass remaining (Figure 2.1). In addition, decay rates ranged from 0.0171 - 0.009 respectively. Kruskal Wallis test concluded that decay rates were not significantly different by leaf type ($T_{3, 12} = 5.4485$, $p = 0.1412$)

Microbial communities

Non-metric multidimensional scaling (nMDS) ordination plots show microbial communities cluster by region (Figure 2.2) and by leaf type (Figure 2.3). Further, ANOSIM revealed that bacterial communities significantly differed by region (R= 0.779, p=0.001) and leaf type (R= 0.657, p= 0.001). In addition, fungal communities significantly differed by region (R= 0.896, p= 0.001) and leaf type (R= 0.682, p= 0.001) (Table2.1). Pairwise analysis determined that microbial communities colonizing individual leaf types were significantly different from each other (Table 2.2).

Microbial communities on specific leaf types also differed depending on the amount of time leaf packs remained in the stream (Table 2.3). Bacterial communities were significantly different over time intervals for *A. rubrum* (R= , p=), *Q. Prinus* and *D. excels* (R= , p=). Fungal communities were significantly different over time for *A. rubrum* (R= , p=), *C. Schreberiana* and *D. excels* (R= , p=). Pairwise analysis for each leaf type are presented in Table 2.4). Despite significant differences in microbial community composition, diversity indices did not differ significantly between leaf types for both bacteria and fungi (Table 2.5).

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DISCUSSION

The overall objective of this study was to quantify microbial community composition during leaf breakdown in two regions using four different leaf types. The results of this experiment supported my hypothesis that microbial communities colonizing leaf detritus differed by study region and by leaf type. These differences in microbial communities were apparent throughout leaf breakdown despite time intervals. Leaf types for this study were chosen due to their abundance in the riparian zones of the areas studied. To a lesser extent, they were chosen because of their differences in initial leaf chemistry as determined by literature estimates. Because of the use of different leaf type, we are only able to draw broad conclusions from the differences in microbial communities between regions.

Contrary to our expectations, leaf decay rates did not differ significantly between the four different leaf types. According to the classification system developed by Petersen and Cummins (1974), leaf decomposition was fast (>0.010) for all leaf types except *Q. prinus* which was classified as slow (<0.005). Leaf decay rates for temperate and tropical leaf types fall within the range of those found in the literature (Webster et al. 2001, Wright and Covich 2005, Bobeldyk and Ramírez 2007, Kominoski et al. 2007, Greenwood et al. 2007).

Initial leaf chemistry has been found to be relatively similar between leaves from different regions. Further, lignin concentrations have been found to be negatively correlated with litter decay rates in both temperate and tropical regions (Campbell and Fuchshuber 1995, Ardon et al. 2009). When lignin concentrations from the literature were compared to mean decay rate from our study, there was a negative correlation. *Q. prinus* exhibited the slowest decay rates in our study and had the highest concentration of lignin out of all four leaf types. In contrast, *D.*

excelsa exhibited the fastest decay rates in our study and had the lowest concentration of lignin out of all four leaf types (Fonte and Schowalter 2004, Kominoski et al. 2007). While the direct relation between lignin concentrations and our decay rates cannot be analyzed, our data demonstrate similar trends to those found in the literature.

Differences in leaf chemistry may drive microbial colonization similarly to the colonization of shredding invertebrates. Bacteria and fungi are both successful at breaking down leaves, however, they have been found to break down different types of leaf constituents more efficiently (Wymore et al. 2013). Romani et al. (2006) investigated enzyme activity of bacteria and fungi on *Phragmites* leaves and found that fungi were better at breaking down plant polymers (i.e., lignin, cellulose, and hemicellulose) and bacteria were better at processing simple molecules (i.e., C, N, and P). Although it is likely that microbes colonize leaves in a random manner, those that can metabolize leaf constituents may successfully attach and continue to process leaves.

In addition, bacteria and fungi communities were assessed for each leaf type between the four time intervals (7, 21, 35, 49). Differences in bacteria and fungi communities were observed for all leaf types except fungi communities on *C. schreberiana* and bacteria communities on *Q. prinus*. We expected microbial succession to occur because leaf material is continually processed and over time leaf constituents are released (Abelho 2001). However, nMDS ordinations depict a weak trend between intervals despite significant differences from ANOSIM. The weak trends in time could be explained by the high variability found between replicates or the short time scale used in this study. It is also possible that leaf types that exhibit lower decay rates (such as *Q. prinus*) require more time to adequately see distinct patterns in succession over time.

While leaf type was important, in determining microbial community composition, the results of this experiment did not support my hypothesis that richness and Shannon's Diversity index would differ between leaf types. This suggests that despite similarities in the number of microbial species, each region may have a unique suite of microbes colonizing leaves over different stages of decay. In addition, the lack of relationship between diversity (richness and Shannon's Diversity) and decay rates supports the idea that many microbial communities have high levels of redundancy (Lawton 1994, Hunt and Wall). This can be important in ecosystems exposed to high frequency of disturbance (Cardinale et al. 2000, Wellnitz, and Poff 2001). Those community members that are able to persist or recover quickly during disturbance events can continue to function (i.e., breakdown constituents, cycle nutrients) within the system (Lake 2000). Because tropical stream systems, such as in Puerto Rico, can exhibit 'flashy' hydrographs (Boulton et al. 2008), it may be beneficial for communities to have high levels of redundancy to recover from frequent disturbances, such as storm events. Future studies could assess the functional role of microbes that are present within these 'disturbance-driven' systems in order to better understand the potential effects of microbial community redundancy in ecosystem processes.

In summary, this study is unique in the fact that few studies have used molecular techniques to assess microbial communities during the initial stages of leaf breakdown in a temperate and a tropical stream. We found differences in microbial communities colonizing leaf litter between the two different regions and the four leaf types. While differences in leaf chemistry is a more likely driver of microbial communities, differences in the microbial communities between regions may be due to regional differences in the species pool that are set by geographic boundaries. This study establishes important information in the understanding of

organic matter processing in temperate and tropical regions. Results from this study suggest that nutrients from leaves are being processed quickly by colonizing communities and are ultimately retained within the headwaters in each region. Future studies could improve upon these findings by exploring next generation sequencing to link functionality and species origin to microbes associated with leaf litter processing in stream ecosystems.

Table 2.1. Analysis of Similarities (ANOSIM) to test for differences by leaf type (A. *rubrum, Q.*

prinus, C. schreberiana, and D. excelsa) and by region (North Carolina and Puerto Rico).

Bacterial and fungal communities were analyzed separately for each factor. Global R values

determine differences between and within groups and range from -1 to +1. (* indicated

significant values $p < 0.05$).

Factor	Bacteria		Fungi	
	Global	P	Global	P
Region	0.779	0.001*	0.896	0.001*
Leaf Type	0.657	0.001*	0.682	0.001*

Table 2.2. Analysis of Similarities (ANOSIM) pairwise results to test for differences between leaf types (A. *rubrum, Q. prinus, C. schreberiana, and D. excelsa*) Bacterial and fungal communities were analyzed separately between leaf types. R statistic values determine differences between groups and range from -1 to +1. (* indicated significant values $p < 0.05$).

Groups	Bacteria		Fungi	
	R	P	R	P
A. rubrum, C. schreberiana	0.833	0.001*	0.864	0.001*
A. rubrum, D. excelsa	0.734	0.001*	0.951	0.001*
A. rubrum, Q. prinus	0.266	0.001*	0.399	0.001*
C. schreberiana, D. excelsa	0.439	0.001*	0.115	0.044*
C. schreberiana, Q. prinus	0.968	0.001*	0.819	0.001*
D. excelsa, Q. prinus	0.815	0.001*	0.902	0.001*

Table 2.3. Analysis of Similarities (ANOSIM) to test for differences by time (7, 21, 35, and 49

days) for each individual leaf type (A. *rubrum, Q. prinus, C. schreberiana, and D. excelsa*).

Bacteria and fungi communities were analyzed separately for each leaf type. Global R values

determine differences between and within groups and range from -1 to +1. (* indicated

significant values $p<0.05$).

Time	Bacteria		Fungi	
	R	P	R	P
A. rubrum	0.646	0.001*	0.195	0.017*
Q. prinus	0.463	0.001*	0.082	0.224
C. schreberiana	0.26	0.077	0.766	0.004*
D. excelsa	0.41	0.003*	0.293	0.012*

1   Table 2.4. Analysis of Similarities (ANOSIM) pairwise results to test for differences by time (7, 21, 35, and 49 days) for each

2   individual leaf. Bacteria and fungi communities were analyzed separately for each leaf type. R statistic values determine differences

3   between groups and range from -1 to +1. (* indicated significant values p<0.05).

4

5

Fungi

Groups	A. rubrum		Q. prinus		C. schreberiana		D. excelsa	
	R	P	R	P	R	P	R	P
07, 21	0.094	0.257	0.185	0.257	N/A	N/A	N/A	N/A
07, 35	0.219	0.086	0.188	0.143	N/A	N/A	N/A	N/A
07, 49	0.24	0.086	0.531	0.057	N/A	N/A	N/A	N/A
21, 35	0.229	0.086	0.111	0.257	0.958	0.029*	0.296	0.143
21,49	0.188	0.114	0.093	0.371	1	0.029*	0.365	0.029*
35, 49	0.125	0.229	0.094	0.257	0.042	0.486	0.278	0.114

Bacteria

Groups	A. rubrum		Q. prinus		C. schreberiana		D. excelsa	
	R	P	R	P	R	P	R	P
07, 21	0.073	0.286	0.111	22.9	N/A	N/A	N/A	N/A
07, 35	0.698	0.029*	0.611	0.029*	N/A	N/A	N/A	N/A
07, 49	1	0.029*	0.944	0.029*	N/A	N/A	N/A	N/A
21, 35	0.833	0.029*	0.365	0.086	0.5	11.4	0.37	0.057
21, 49	0.927	0.029*	0.885	0.029*	0.463	0.057	0.573	0.029*
35, 49	0.385	0.029*	0.417	0.057	-0.094	0.657	0.352	0.057

Table 2.5. Mean Richnesss (S) and Shannon's Diversity (H') for bacteria and fungi colonizing four the leaf types (A. *rubrum, Q. prinus, C. schreberiana, and D. excels*a) during field experiments. Values are presented with ± Standard Error.

	Bacteria		Fungi	
	S	H'	S	H'
	(± SE)	(± SE)	(± SE)	(± SE)
A. rubrum	24.688	2.931	11.6875	1.847
	(±0.123)	(±0.040)	(±0.109)	(±0.098)
Q. prinus	25.533	2.961	14.667	2.175
	(±0.150)	(±0.064)	(±0.126)	(±0.080)
C. schreberiana	28.455	3.112	16.917	2.234
	(±0.126)	(±0.029)	(±0.191)	(±0.149)
D. excelsa	23.636	2.761	13.700	2.027
	(±0.442)	(±0.201)	(±0.234)	(±0.139)
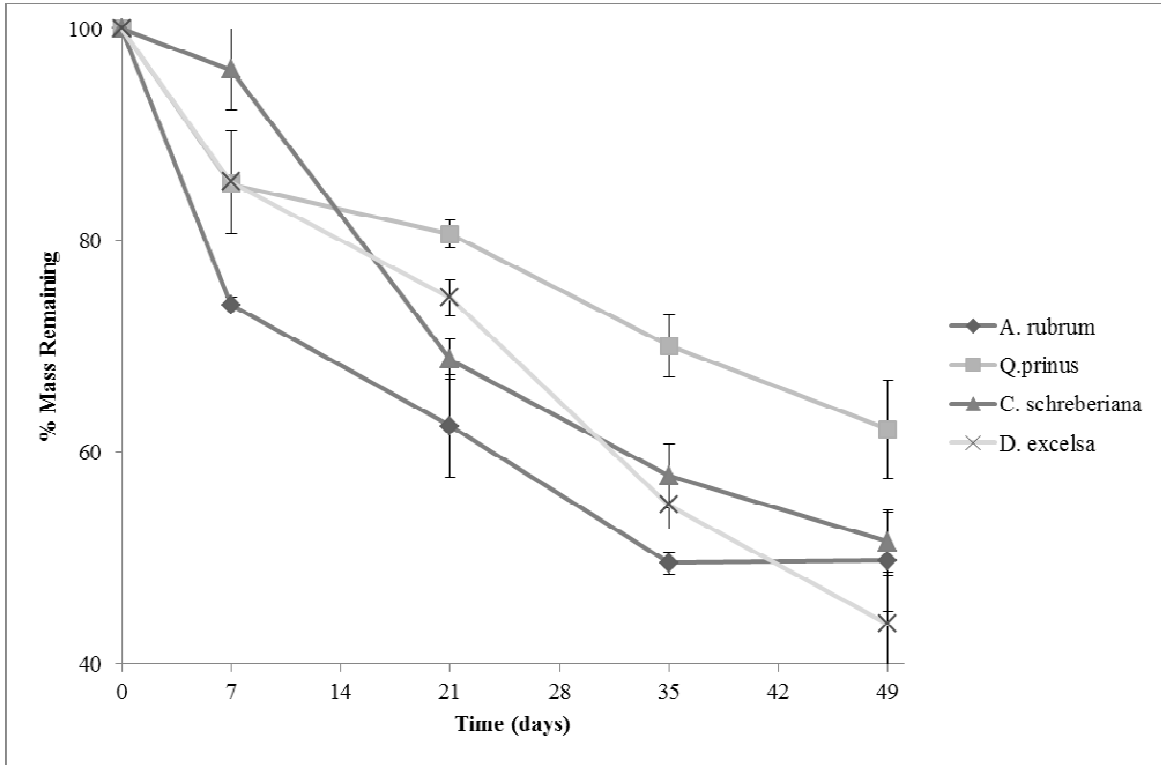
Figure 2.1. Mean percent mass remaining for all four leaf types (*A. rubrum, Q. prinus, C. schreberiana, and D. excelsa*) at four time intervals. *A. rubrum, Q. prinus,* were deployed in North Carolina *and C. schreberiana, and D. excelsa* were deployed in Puerto Rico. Error bars represent one standard error (n = 4).
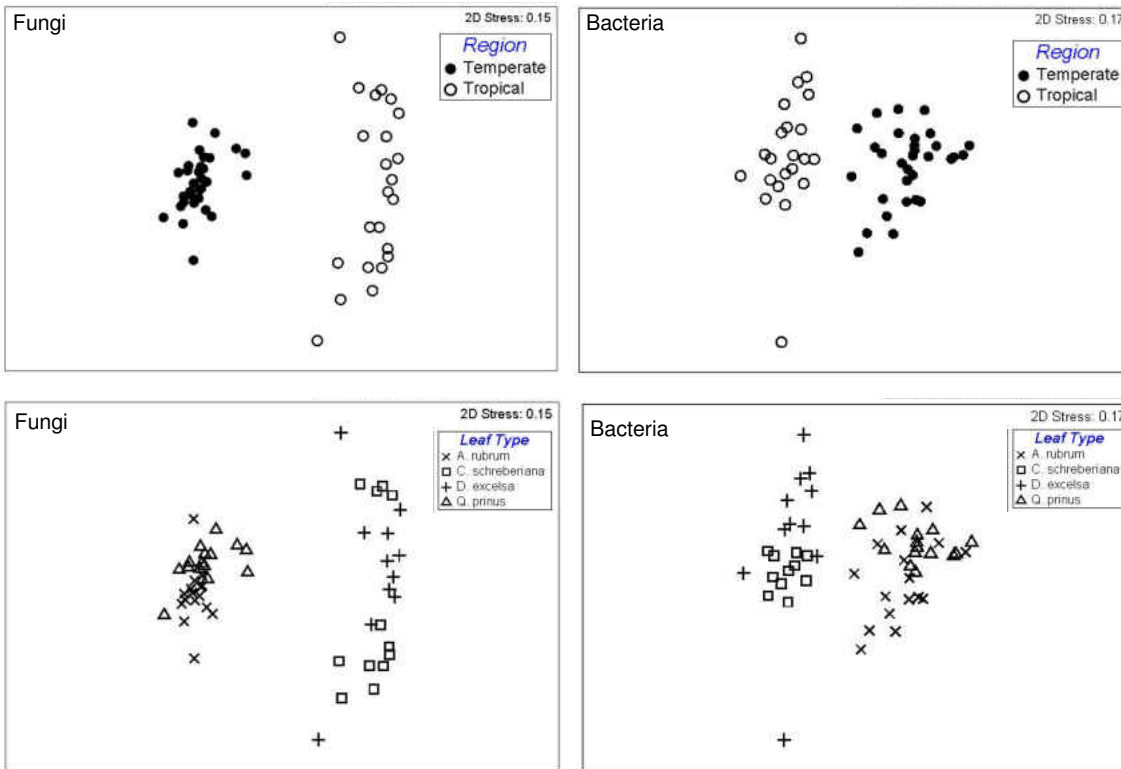
Figure 2.2. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial and fungal communities among two regions and four leaf types. Each point represents the square root of relative abundance of all TF peaks in a sample. In plots A and B, North Carolina is represented by closed circles and Puerto Rico is represented by open circles. In plots C and D, *A. rubrum* is represented by X; *Q. prinus* is represented by triangles; *C. schreberiana* is represented by squares; and *D. excelsa* is represented by pluses.
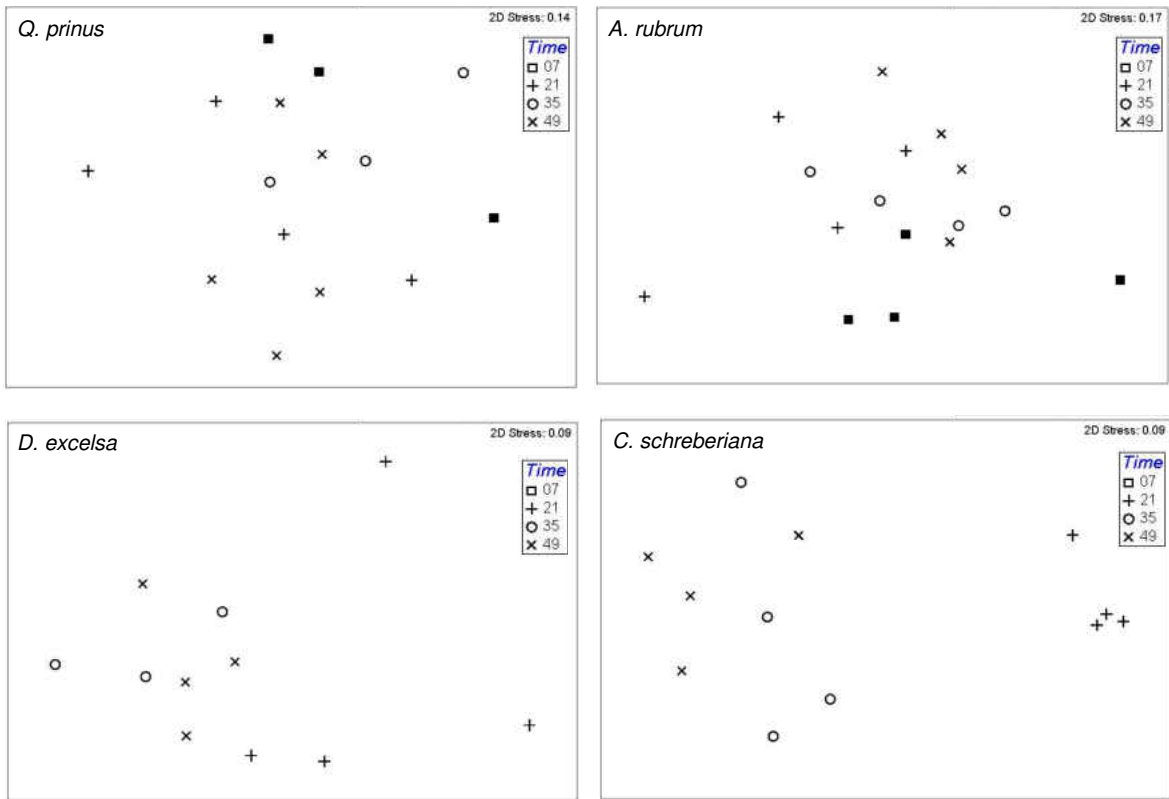
Figure 2.3. Non-metric multidimensional scaling (nMDS) ordination comparing fungal communities among four time intervals for all four leaf types. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, day 7 is represented by squares, day 21 is represented by pluses; day 35 is represented by circles; and day 49 is represented by X.
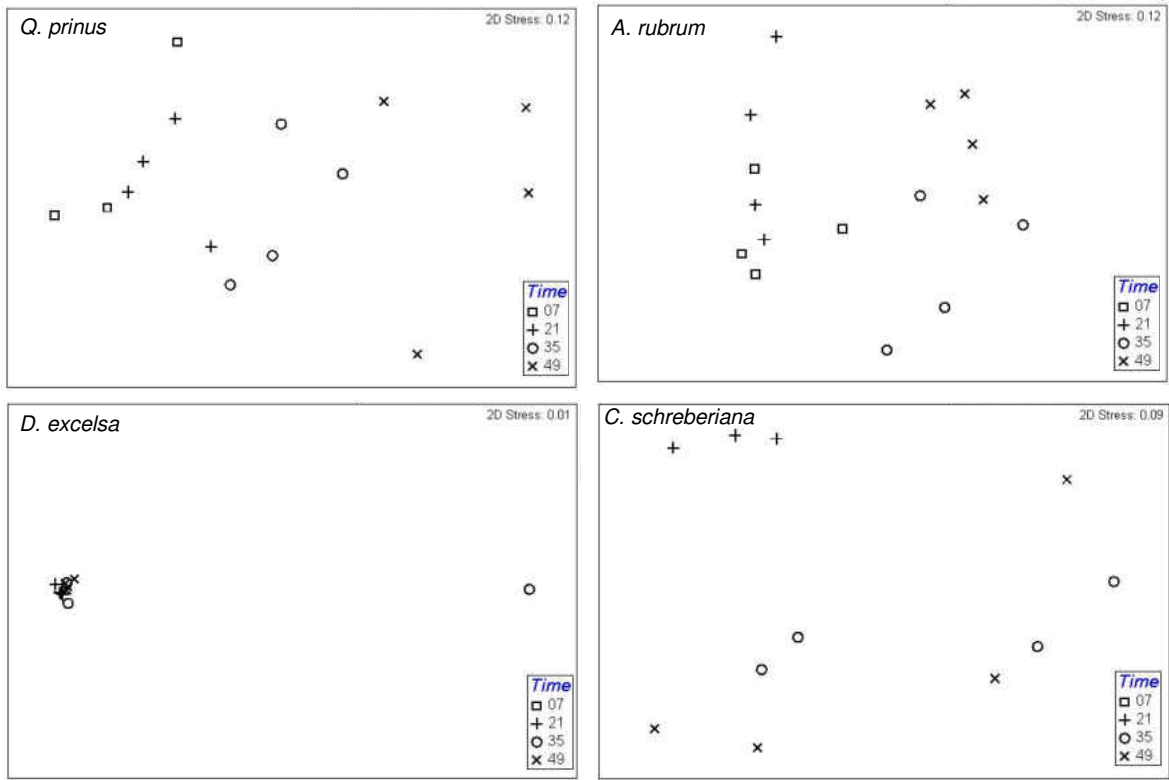
Figure 2.4. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial communities among four time intervals for all four leaf types. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, day 7 is represented by squares, day 21 is represented by pluses; day 35 is represented by circles; and day 49 is represented by X.

CHAPTER 3

An assessment of microbial communities colonizing fallen leaves during early stages of decay

using mesocosms

INTRODUCTION

Organic matter processing is a major ecosystem function that fuels small, forested, headwater streams (Vannote et al. 1980, Cummins et al. 1989, Abelho 2001). Organic matter processing has been intensely studied in streams as an attempt to better understand the mechanisms behind this important ecological process (Petersen and Cummins 1974, Webster et al. 1999). Bacteria and fungi are capable of metabolizing leaf constituents and converting complex compounds into nutrients that can be used by other biota (Barlocher 1985). Furthermore, bacteria and fungi have varying efficiencies at breaking down leaf constituents. Romani et al. (2006) compared the enzymatic capabilities of both bacteria and fungi during leaf breakdown. They found that bacteria were more efficient at breaking down basic elements (i.e., C, N, and P), where as fungi were more efficient at breaking down complex compounds (i.e., lignin, cellulose, and hemicellulose).

By breaking down complex compounds, microbes play an important role in carbon cycling throughout the stream because they make nutrients available to higher trophic levels as compounds are either released or incorporated into microbial tissues (Barlocher 1985, Graca et al. 1993, Wong et al. 1998). Microbes are known to condition leaf substrates in such a way that facilitates consumption by invertebrate consumers, known as shredders (Suberkropp 1992, Webster and Wallace 1996, Rincon and Martinez 2006). Furthermore, some shredding insects, such as caddisfly larvae, are known to preferentially feed upon leaves that have been colonized by fungi (Arsuffi and Suberkropp 1985).

Shredders are also known for deriving much of their nutrition from assimilating microbes rather than the low quality leaf material by selectively feeding on fungal colonized patches (Arsuffi and Suberkropp 1984). Graca et al. (1999) investigated biomass of two freshwater crustaceans after selectively feeding on fungi and found a positive correlation between consumption of fungi and biomass of the isopod *Asellus aquaticus*. However, the amphipod *Gammarus pulex* did not exhibit this response suggesting that each shredder species has specific preference and assimilation efficiencies for this resource. In addition, Chung and Suberkropp (2009) found that fungal biomass can contribute significantly to the growth of a shredding caddisfly, *Pycnopsyche gentilis*. However, this caddisfly had shown to exhibit less selectivity in their feeding than other caddisflies shredders examined.

While many of the studies to date used a finite number of microbes in shredder feeding trials, leaf substrates host a community of microbes that differ in composition throughout breakdown stages. (Arsuffi and Suberkropp 1989). My goals were to use laboratory decomposition trials to determine microbial community diversity on decaying leaves in the presence and absence of invertebrate consumers. In doing so, this study used molecular techniques to address the following objectives: (i) compare microbial communities found on leaf substrates between different leaf types, (ii) compare microbial communities on leaf substrates in the presence and absence of an invertebrate consumer. I hypothesized that microbial community structure would differ between leaf types and if invertebrate consumers were present or absent.

METHODS AND MATERIALS

Study Site

Two dominant riparian leaf species and one species of invertebrate consumer were

collected from each of the Coweeta LTER in North Carolina and the Luquillo Experimental

Forest in Puerto Rico. At the Coweeta LTER, leaf and consumer collections were made at

catchments 53 and 54. At the Luquillo LEF, leaf and consumer collections were made at

Quebrada Gatos. Leaves were collected post abscission, stored at room temperature, and

transported to Georgia Southern University until they were used in mesocosm experiments.

Invertebrate consumers were hand-picked from the stream and transported back to the laboratory

where they were kept in constant temperature chambers before being added to the mesocosms.

Mesocosms

Mesocosms maintained at a constant temperature environmental chamber (Model 3940

Forma Environmental Chamber; Thermo Scientific). Trials were run at two different

temperatures, 15°C and 20°C, to simulate temperatures of the region where the consumers used

in each trial were collected from. All mesocosms contained approximately 300 mL of moderately

hard water (as 100mg/l CaCO3) prepared in the laboratory (Table 3.1) along with one air stone.

Mesocosms were set up with one of four leaf types: *Acer rubrum*(temperate), *Quercus alba*

(temperate) *Dacryodes excelsa* (tropical) or *Cecropia schreberiana* (tropical). Leaves were cut

into leaf disks using a 14 mm cork borer. Twenty leaf disks were separated, weighed and placed

into the appropriate mesocosms (28 per leaf type).

Mesocosms were placed in environmental chamber after the desired temperatures were

obtained (approximately 24 hrs). In order to account for initial leaching and early conditioning,

16 mesocosms (4 replicates of each leaf type) were removed after 7 days. Once removed,

invertebrate consumers were added to 48 mesocosms. For the temperate trials, ran at 15°C,

nymphs of *Tallaperla maria* (a common stonefly shredder found in Coweeta streams) were used

and for the tropical trials, ran at 20°C, nymphs of *Phylloicus pulchrus* (a common caddisfly

shredder found in Luquillo streams) were used. After consumers were added, 32 mesocosms

were removed every 14 days (i.e., 21, 35, 49). This set up was replicated 4 times to include a

total of 112 mesocosms for each temperature trials.

Decay Rates

Ten leaf disks, randomly selected from each mesocosms, were removed after each time interval

(7, 21, 35, and 49), consequently dried at 60°C for 48 h and weighted to the nearest 0.0001g.

Leaf decay rates were estimated using an exponential decay model where k (decay coefficient) is

estimated as the rate of original mass loss from the following equation:

$$k = Ln(OMR\%/100)/t,$$

where OMR% = the percent orginial mass remaining, and t = time in days (Petersen and

Cummins 1974, Benfield 2006).

Microbial Community Analysis

      To assess microbial community structure, environmental DNA was extracted from leaf

material taken from replicate leaf packs using the UltraClean Soil DNA Isolation Kit (MoBio

Laboratories, Inc., Carlsbad, California) following the manufacturers' protocol for maximal

yield.

      Bacteria 16S rDNA was amplified from extracted DNA using primers 8F (5-

AGAGTTTGATCCTGGCTCAG-3) and 907R1 (5- CCGTCAATTCCTTTGAGTTT-3). The

forward primer, 8F was labeled with a blue FAM fluorescence tag. Each reaction had a total of 10 μl and included 1X Apex Taq Master Mix (1.5mM MgCl$_2$, Genesee Scientific, San Diego, California), 1 μM of each primer, 1 μl of DNA and sterilized water was added to reach the final volume. The reactions were performed with an initial denaturation at 94 ℃ (3 min), followed by 30 cycels of denaturation at 94 ℃ for 30s, annealing at 55℃ for 30s and extension at 72 ℃ for 30s, plus a final elongation at 72 ℃ for 5 min. PCR products were confirmed by gel electrophoresis.

Fungal ITS regions were amplified from extracted DNA using primers ITS1-F(5-C TTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3). The forward primer ITS1-F was labeled with a green HEX fluorescence tag. Each reaction had a total of 10 μl and included 1X Apex Taq Master Mix (1.5mM MgCl2, Genesee Scientific, San Diego, California), 1 μM of each primer, 1 μl of DNA and sterilized water was added to reach the final volume. The reactions were performed with an initial denaturation at 94 ℃ (3 min), followed by 35 cycles of denaturation at 94 ℃ for 60s, annealing at 55 ℃ for 60s and extension at 72 ℃ for 60s, plus a final elongation at 72 ℃ for 10 min. PCR products were confirmed by gel electrophoresis.

PCR products of bacterial16S rDNA were digested with HaeIII and MspI and fungal ITS rDNA were digested with HaeIII and RsaI respectively. Bacterial digests included 1 X RE buffer, 0.25 U/μl of each restriction enzyme, 2 ul of PCR product, and sterilized water was added to a final volume of 10 ul. Fungal digests included 1 X RE buffer, 0.25 U/μl of each restriction enzyme, 2 μl of PCR product, and sterilized water was added to a final volume of 10 μl. PCR products were digested at 37 ℃ for 6 h.

Restricted PCR products were loaded on an automated capillary electrophoresis sequencer Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster City, California) to detect terminal restriction fragment length polymorphisms (T-RFLP). One ul digests were mixed with 10 µl of HiDi Formamide (Applied Biosystems, Foster City, California) and 0.5 µl of size standard Liz600 (Applied Biosystems, Foster City, California). Raw T-RF data were detected using GeneMapper ver. 3.7. T-RF peaks were identified from individual T-RF profiles and relative abundance was calculated using peak area. Relative abundance for each sample was arranged and aligned by base pairs (±0.5) using TREEFLAP (Walsh, C. Monash Univ, http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls). Peaks comprising of <1% of total area along with bacterial peaks <100 bp and fungal peaks <50 bp were excluded from the analysis. Bacterial peaks <100 bp were excluded to avoid apparent contamination that was unavoidable in laboratory trails.

Statistical Analyses

Due to the lack of space in the environmental chamber, replicates were divided in half and were run at separate times. Preliminary TF analysis and decay rate calculations suggested that the same treatments that were run at different times were statistically different. Due to these differences, analyses were completed on only half of the replicates from both the temperate (Run 1) and the tropical (Run 3) trials. The replicates chosen for analyses were selected because they had the largest number of samples that had successful T-RFLP results.

Analysis of Variance (ANOVA) was used to test the effects of leaf type on decay rates using statistical package (JMP v. 10.0 SAS Institute Inc. 2010). Microbial community profiles were analyzed using non-metric multidimensional scaling (nMDS) and Analysis of Similarities (ANOSIM). Dissimilar communities were further analyzed with Similarity Percentages

(SIMPER) to determine which TFs were driving community differences. All multivariate analyses, including diversity indices, Bray-Curtis similarity, nMDS, and ANOSIM were conducted using the PRIMER-E v.6 statistical software (Clarke and Gorley 2006

RESULTS

Temperate Laboratory Trials

After the 7-day conditioning period during the temperate trials, percent original mass remaining ranged from 93-72% with *A. rubrum* decaying the fastest and *Q. prinus* decaying the slowest. By the end of the 49 day experiment, percent original mass remaining in mesocosms where consumers were absent ranged from 91-69% with *A. rubrum* losing the most mass and *Q. prinus* losing the least amount of mass. By the end of the 49 day experiment, percent original mass remaining in mesocosms where consumers were present ranged from 88-57% with *A. rubrum* losing the most mass and *C. schreberiana* losing the least amount of mass. Percent original mass remaining over time is depicted in Figure 3.1 and associated decay rates are depicted in Figure 3.3A.

Analysis of Similarity (ANOSIM) revealed that bacterial communities significantly differed by overall leaf type (Global R=0.133, p=0.001) and consumer (Global R=0.065, p=0.047) and for fungi by leaf type (Global R=0.306, p=0.001) (Table 3.2). Bacterial communities differed on *Q. prinus* between consumer and *C. schreberiana* between time intervals but not for other leaf types for either consumer treatments or time intervals (Table 3.5). Fungal communities differed on *C. schreberiana* between time intervals but not for other leaf types for either consumer treatments or time intervals (Table 3.5).

Tropical Laboratory Trials

After the 7-day conditioning period during the temperate trials, percent original mass remaining ranged from 94-68% with *A. rubrum* losing the most mass and *C. schreberiana* losing the least amount of mass. By the end of the 49 day experiment, percent organic matter remaining in mesocosms where consumers were absent ranged from 86-65% with *A. rubrum* losing the most mass and *Q. prinus* losing the least amount of mass. By the end of the 49 day experiment, percent organic matter remaining in mesocosms where consumers were present ranged from 88-57% with *A. rubrum* losing the most mass and *C. schreberiana* losing the least amount of mass (Figures 3.2 and 3.3B).

Analysis of Similarity (ANOSIM) revealed that bacteria communities significantly differed by overall leaf type (Global R=0.153, p=0.001) and consumer (Global R=0.11, p=0.003) and for fungi by leaf type (Global R=0.33, p=0.001) (Table 3.2Bacterial communities differed on *Q. prinus* and *D. excelsa* between consumer treatments but not for other leaf types for either consumer treatments or time intervals Table 3.6). Fungal communities differed on *A. rubrum* between time intervals but not for other leaf types for either consumer treatments or time intervals (Table 3.6). Non-metric multidimensional scaling (nMDS) ordination plots associated with these ANOSIM values are depicted in Figures 3.14, and 3.15.

DISCUSISON

The main goal of this study was to investigate microbial communities during leaf breakdown by recreating stream dynamics in a laboratory setting. While molecular analyses indicated that microbial communities were present at each time interval, my laboratory trials were unsuccessful. Between different runs in the environmental chamber, a different batch of leaves were collected and dried. Even though separate laboratory trials had the same type and

number of replicates, ANOSIM analysis classified microbial communities as being significantly different.

After selecting single chamber runs (n=2), leaf decay and microbial community trends were able to be assessed. As expected, over time leaves lost mass for each leaf type. Decay rates for the laboratory trials were faster in mesocosms where *T. maria* was present. This suggests that *T. maria* was capable of further breaking down leaf discs in addition to the microbes colonizing leaf discs. However, in tropical laboratory trials, decay rates for *C. schreberiana* and *D. excelsa* appeared faster for those mesocosms where *P. pulchrus* was absent. *P. pulchrus* utilizes leaf materials for case building but because both tropical leaf species had slow decay rates, *P. pulchrus* may not have been able to construct cases due to the toughness of the leaves (Ardon et al. 2008). In addition, tropical streams have been described as having a paucity of insect shredders and may rely on microbes as the major colonizing consumers of leaf detritus (Irons et al. 1994).

Analysis on individual runs supported my hypothesis that leaf type and consumer influenced community composition of microbial communities during leaf breakdown. Pairwise analysis suggested that not all leaf types were significantly different from one another but this lack of significance may be due to the low sample number used to calculate ANOSIM values. Statistical tests could not be run to determine the influence of microbial diversity on decay rates due to small samples sizes. However, data from field experiments (chapter 2) suggest that richness and diversity of microbial communities may not be the driving force of decay rates.

A visual comparison of the microbial richness of this laboratory study compared to the field study showed that laboratory communities were less rich (Chapter 2). A possible explanation for different numbers of microbes may be due to the lack of nutrients. Leaf material

is a low quality material with high C:N concentrations (Cummins et al 1989, Gessner et al. 1999, Abelho et al 2001). Studies have used nutrient additions to support microbial communities during the entire process of leaf breakdown (Duarte et al. 2006) In a study investigating the influence of different nutrient levels, Gulis et al. (2008) found higher microbial respiration and biomass in streams with high nutrient concentrations. Therefore, this study may have supported a higher diversity of microbes if stream water would have been used in the mesocosms. Because the mesocosms lacked ambient nutrient levels, microbes may have been far too nutrient limited to process leaf litter efficiently, stimulate microbial activity, and ultimately explain the low numbers of bacteria and fungi found on leaves (Suberkropp et al. 2010).

In my study, I assumed that terrestrial microbial communities present would be quickly replaced by aquatic hyphomycetes due to the inability of terrestrial fungi to adequately function in aquatic ecosystems (Graca and Ferreira 1995). However, since microbial communities were shown to be significantly different, initial terrestrial colonizers may influence the succession of aquatic microbes more than was previously expected. Microbes compete for resources and space and therefore terrestrial fungi may have inhibited other fungi from adequately colonizing leaves in mesocosm laboratory trials (Mille-Lindblom and Tavernik 2003). Another reason microbial community dynamics may have been different is because there weren't adequate aquatic hyphomycetes present to successfully replace terrestrial fungi. As a result, nutrients and initially inoculating leaves with aquatic hyphomycetes are important factors when developing laboratory mesocosms to study microbial communities.

This study stresses the importance of studying microbial communities in the field in addition to the laboratory. Although many researchers have successfully cultured microbes, it is also important to assess in stream interactions of microbes. It has been suggested that the number

and species of microbes have been vastly underestimated in ecosystems (Barlocher 2007) and therefore makes it increasingly difficult to study such dynamic interactions within a laboratory setting. With such great uncertainty into the ecology of microbes, field studies should be used in combination to laboratory trials whenever resources allow.

In summary, this study addresses two important factors on the influence of microbial communities found on decaying leaves. Leaf type and consumer were shown to influence microbial communities throughout breakdown. Although the laboratory set up lacked the necessary variables and power to make concrete conclusions, results are similar to those found during the field experiment (Chapter 2).

Table 3.2: Analysis of Similarities (ANOSIM) to test for differences by leaf type (A. *rubrum, Q.*

prinus, C. schreberiana, and D. excelsa) and by consumer (absent and present). Bacterial and

fungal communities were analyzed separately for each factor. Global R values determine

differences between and within groups and range from -1 to +1. (* indicated significant values

$p < 0.05$).

| | | Bacteria | | Fungi | |
	Run	Global R	P Value	Global R	P Value
Leaf Type	1	0.133	0.001*	0.306	0.001*
Consumer		0.065	0.047*	0.041	0.091
Leaf Type	2	0.13	0.001*	0.052	0.046*
Consumer		0.091	0.026*	0.084	0.9*
Leaf Type	3	0.153	0.001*	0.33	0.001*
Consumer		0.11	0.003*	-0.014	0.666
Leaf Type	4	0.338	0.001*	0.222	0.001*
Consumer		0.019	0.273	0.01	0.337

Table 3.3. Analysis of Similarities (ANOSIM) pairwise results to test for differences between leaf types (A. *rubrum, Q. prinus, C. schreberiana, and D. excelsa*). Depicted below are differences in bacteria communities between leaf types in all four laboratory trials. R statistic values determine differences between groups and range from -1 to +1. (* indicated significant values $p<0.05$).

Bacteria

Groups	Run 1 - Temperate		Run 2 - Temperate		Run 3 - Tropical		Run 4 - Tropical	
	R	P	R	P	R	P	R	P
A. rubrum, Q. prinus	0.214	0.001*	0	0.463	0.106	0.05*	0.348	0.001*
A. rubrum, C. schreberiana	0.166	0.001*	0.263	0.001*	0.32	0.003*	0.296	0.001*
A. rubrum, D. excelsa	0.187	0.002*	0.057	0.08	0.091	0.062	0.064	0.079
Q. prinus, C. schreberiana	-0.009	0.523	0.243	0.003*	0.231	0.001*	0.448	0.001*
Q. prinus, D. excelsa	0.059	0.13	0.026	0.261	0.07	0.094	0.516	0.001*
C. schreberiana, D. excelsa	0.123	0.016*	0.217	0.001*	0.102	0.027*	0.412	0.001*

Table 3.4. Analysis of Similarities (ANOSIM) pairwise results to test for differences between leaf types (A. *rubrum, Q. prinus, C. schreberiana, and D. excelsa*). Depicted below are differences in fungi communities between leaf types in all four laboratory trials. R statistic values determine differences between groups and range from -1 to +1. (* indicated significant values p<0.05).

Fungi Groups	Run 1 - Temperate		Run 2 -Temperate		Run 3 -Tropical		Run 4 -Tropical	
	R	P	R	P	R	P	R	P
A. rubrum, Q. prinus	0.175	0.006	-0.042	0.816	0.208	0.003*	0.248	0.002*
A. rubrum, C. schreberiana	0.552	0.001*	0.046	0.149	0.24	0.004*	0.077	0.045*
A. rubrum, D. excelsa	0.43	0.002*	0.002	0.452	0.166	0.006*	0.048	0.122
Q. prinus, C. schreberiana	0.245	0.001*	0.052	0.15	0.647	0.001*	0.394	0.001*
Q. prinus, D. excelsa	0.249	0.003*	0.088	0.047*	0.597	0.001*	0.404	0.001*
C. schreberiana, D. excelsa	0.241	0.004*	0.164	0.005*	0.107	0.039*	0.215	0.002*

Table 3.5. Analysis of Similarities (ANOSIM) to test for differences by time (7, 21, 35, and 49 days) and consumer (absent and present) for each individual leaf type (A. *rubrum, Q. prinus, C. schreberiana, and D. excelsa*). Bacteria and fungi communities were analyzed separately for each leaf type. Results are depicted for temperate laboratories run at 15 ° C with stonefly *Tallaperla maria* shredders. Global R values determine differences between and within groups and range from -1 to +1. (* indicates significant values $p<0.05$).

Bacteria		*A. rubrum*		*Q. prinus*		*C. schreberiana*		*D. excelsa*	
		R	P	R	P	R	P	R	P
	Consumer	0.073	0.188	0.024	0.036*	0.173	6.9	0.178	0.089
	Time	0.091	0.187	-0.098	0.782	0.268	2.3*	-0.044	61.9

Fungi		*A. rubrum*		*Q. prinus*		*C. schreberiana*		*D. excelsa*	
		R	P	R	P	R	P	R	P
	Consumer	0.026	0.305	-0.073	0.715	0.004	0.46	0.159	0.159
	Time	0.195	0.013*	0.023	0.404	0.258	0.038*	-0.162	0.821

Table 3.6. Analysis of Similarities (ANOSIM) to test for differences by time (7, 21, 35, and 49 days) and consumer (absent and present) for each individual leaf type (A. *rubrum, Q. prinus, C. schreberiana, and D. excelsa*). Bacteria and fungi communities were analyzed separately for each leaf type. Results are depicted for tropical laboratories run at 20 ° C with caddisfly *Phylloicus pulchrus* shredders. Global R values determine differences between and within groups and range from -1 to +1. (* indicates significant values p<0.05).

Bacteria

	A. rubrum		*Q. prinus*		*C. schreberiana*		*D. excelsa*	
	R	P	R	P	R	P	R	P
Consumer	-0.007	0.376	0.248	0.035*	0.109	0.154	0.239	0.026*
Time	0.104	0.229	0.026	0.395	-0.012	51.8	-0.016	50

Fungi

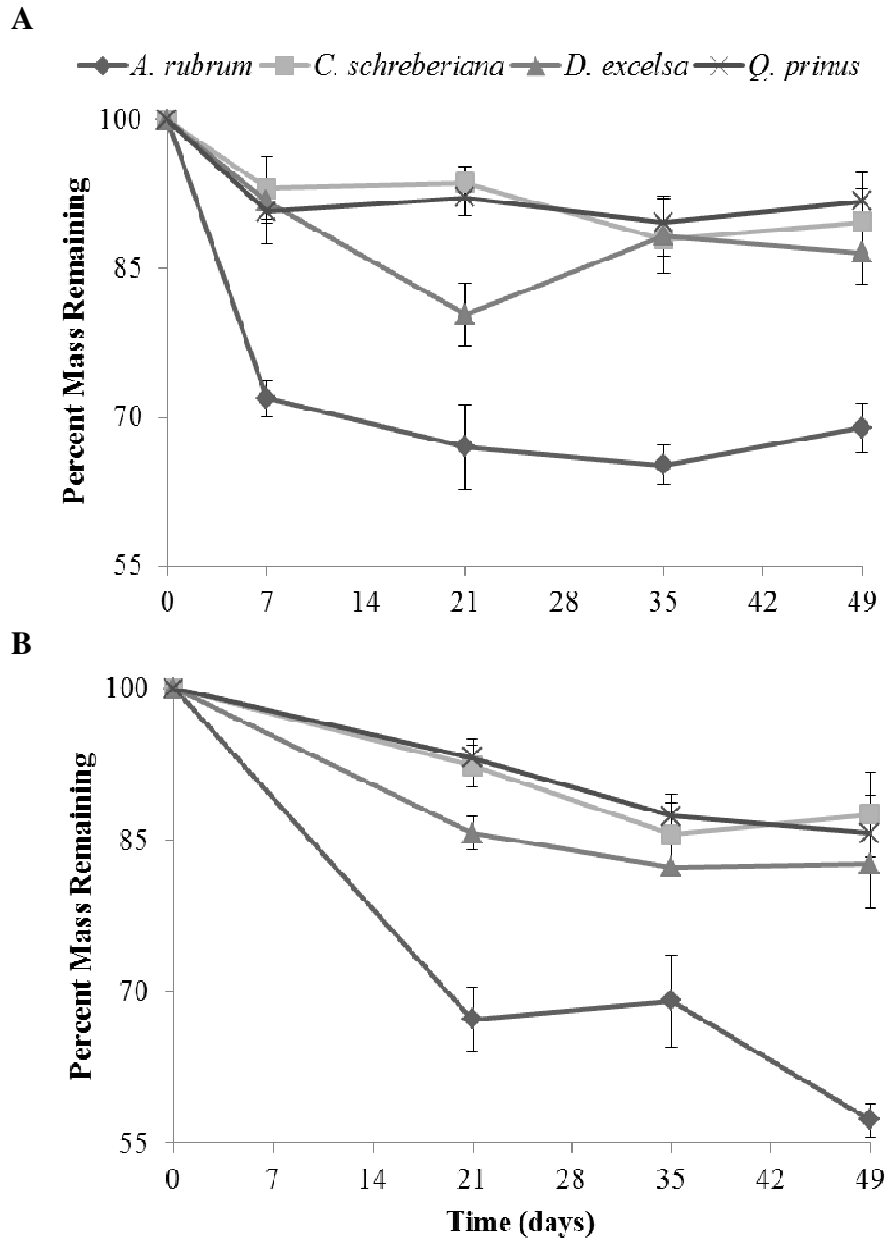|	*A. rubrum*		*Q. prinus*		*C. schreberiana*		*D. excelsa*	
	R	P	R	P	R	P	R	P
Consumer	-0.087	0.78	0.147	0.094	-0.083	0.711	-0.108	0.79
Time	0.279	0.023*	0.028	0.397	0.125	0.215	0.131	0.179

Figure 3.1: Mean percent mass remaining for leaves in temperate laboratory trials run at 15 ° C. (A) Percent mass remaining in mesocosms without shredders and (B) percent mass remaining in mesocosms with shredders (*Tallaperla maria*). Shredders were only added to mesocosms after 7 days and therefore the first data point for these mesocosms is day 21. Error bars represent one standard error (n = 4).
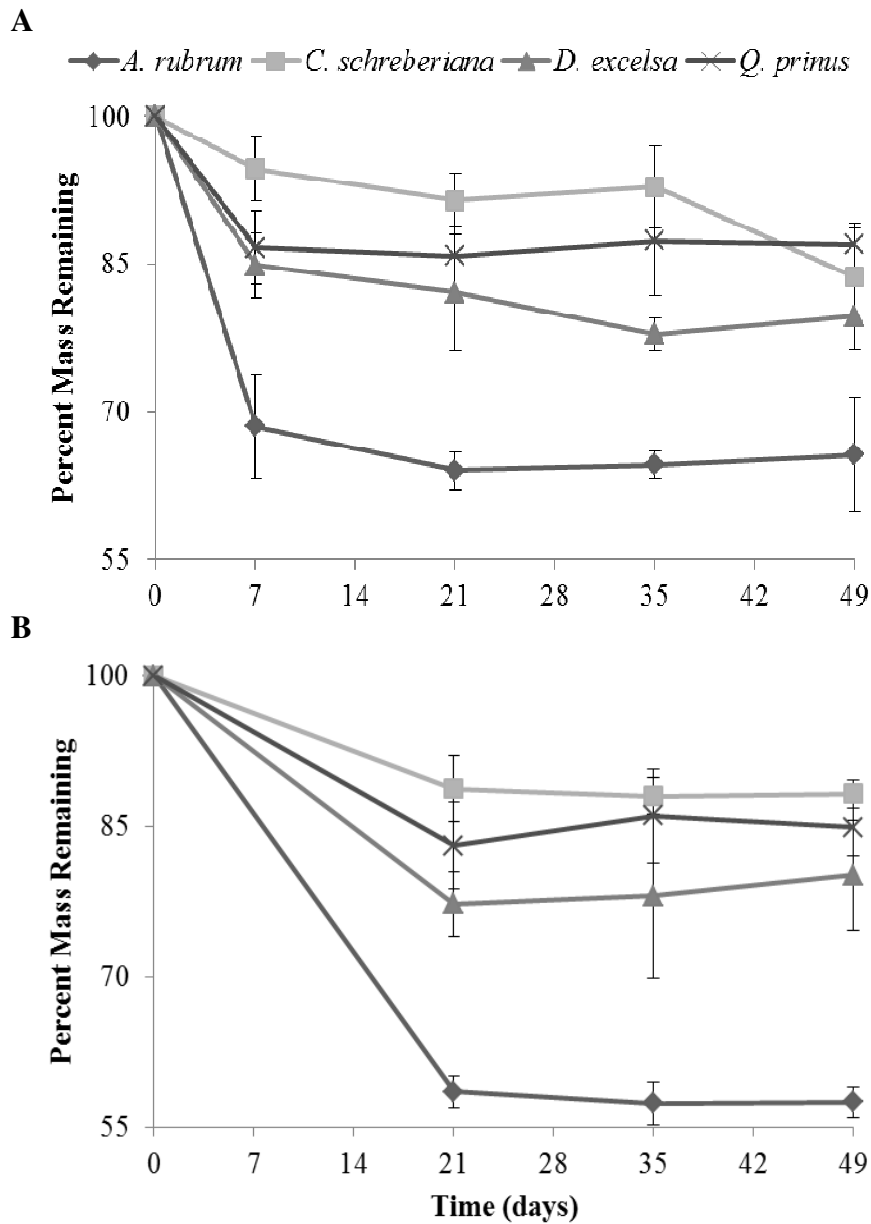
Figure 3.2: Mean percent mass remaining for leaves in tropical laboratory trials run at 20 ° C.

(A) percent mass remaining in mesocosms without shredders and (B) percent mass remaining in

mesocosms with shredders (*Phylloicus pulchrus*). Shredders were only added to mesocosms after

7 days and therefore the first data point for these mesocosms is day 21. Error bars represent one
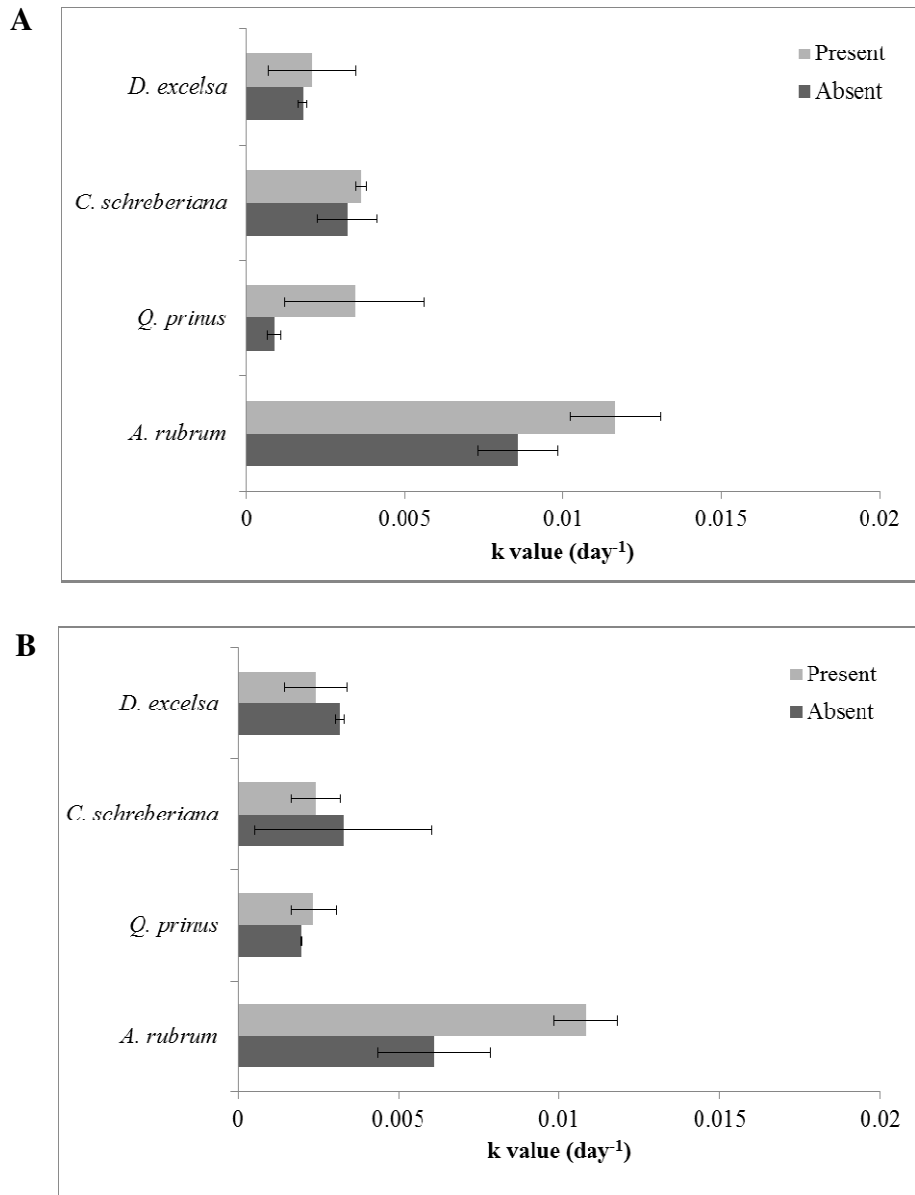
standard error (n = 4).

Figure 3.3. Leaf decay rates (k values) for A. *rubrum, Q. prinus, C. schreberiana, and D. excels*a in laboratory mesocosms. Error bars represent one standard error (n=4). (A) Represents decay rates for temperate laboratory trials and (B) represents decay rates for tropical laboratory trials.
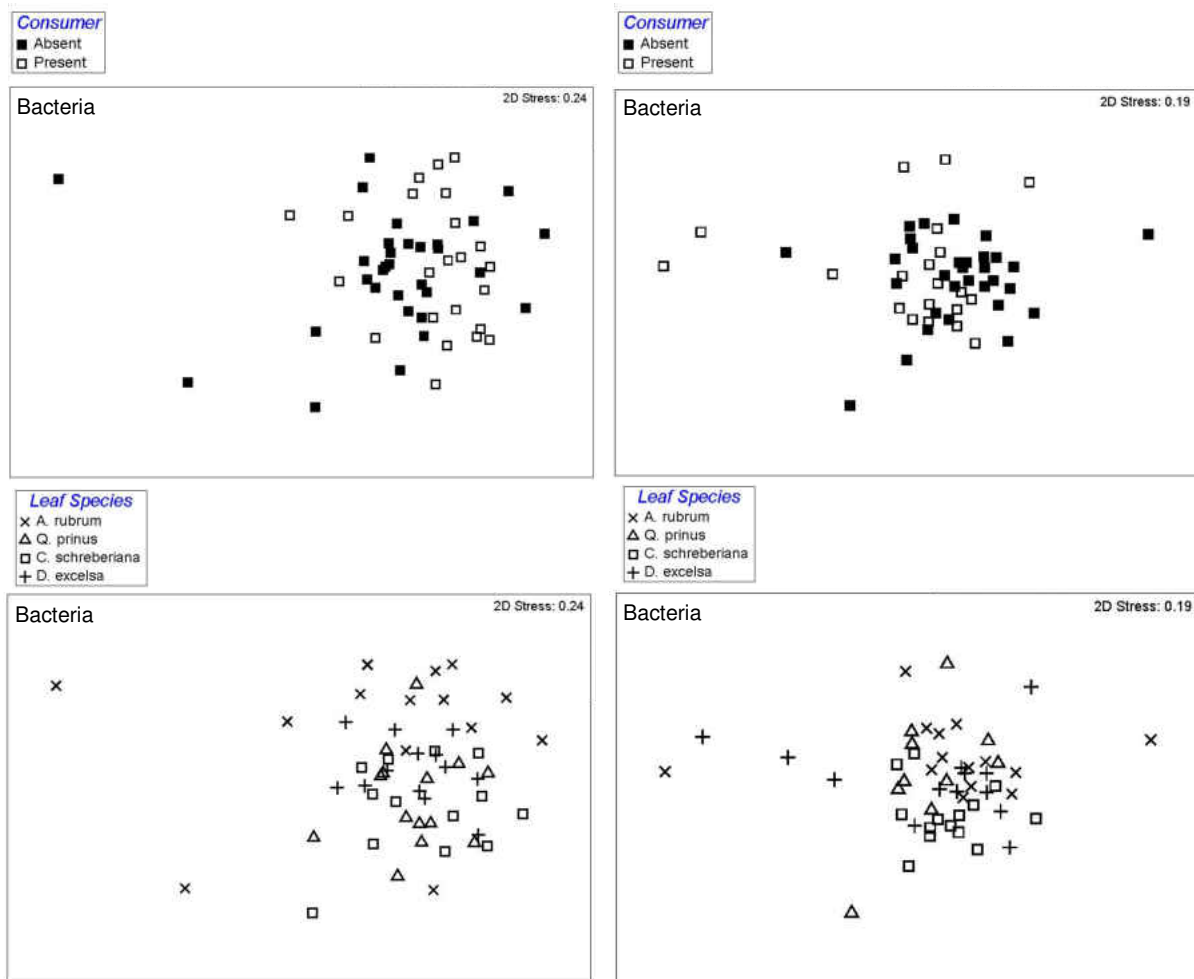
Figure 3.4. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial communities among two consumer treatments (absent and present) and four leaf types (*A. rubrum, Q. prinus, C. schreberiana, and D. excelsa*) for temperate laboratory trails (Run 1 and Run 2). Each point represents the square root of relative abundance of all TF peaks in a sample. In the top plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares. In the bottom plots, *A. rubrum* is represented by X's; *Q. prinus* is represented by triangles; *C. schreberiana* is represented by squares; and *D. excelsa* is represented by pluses.
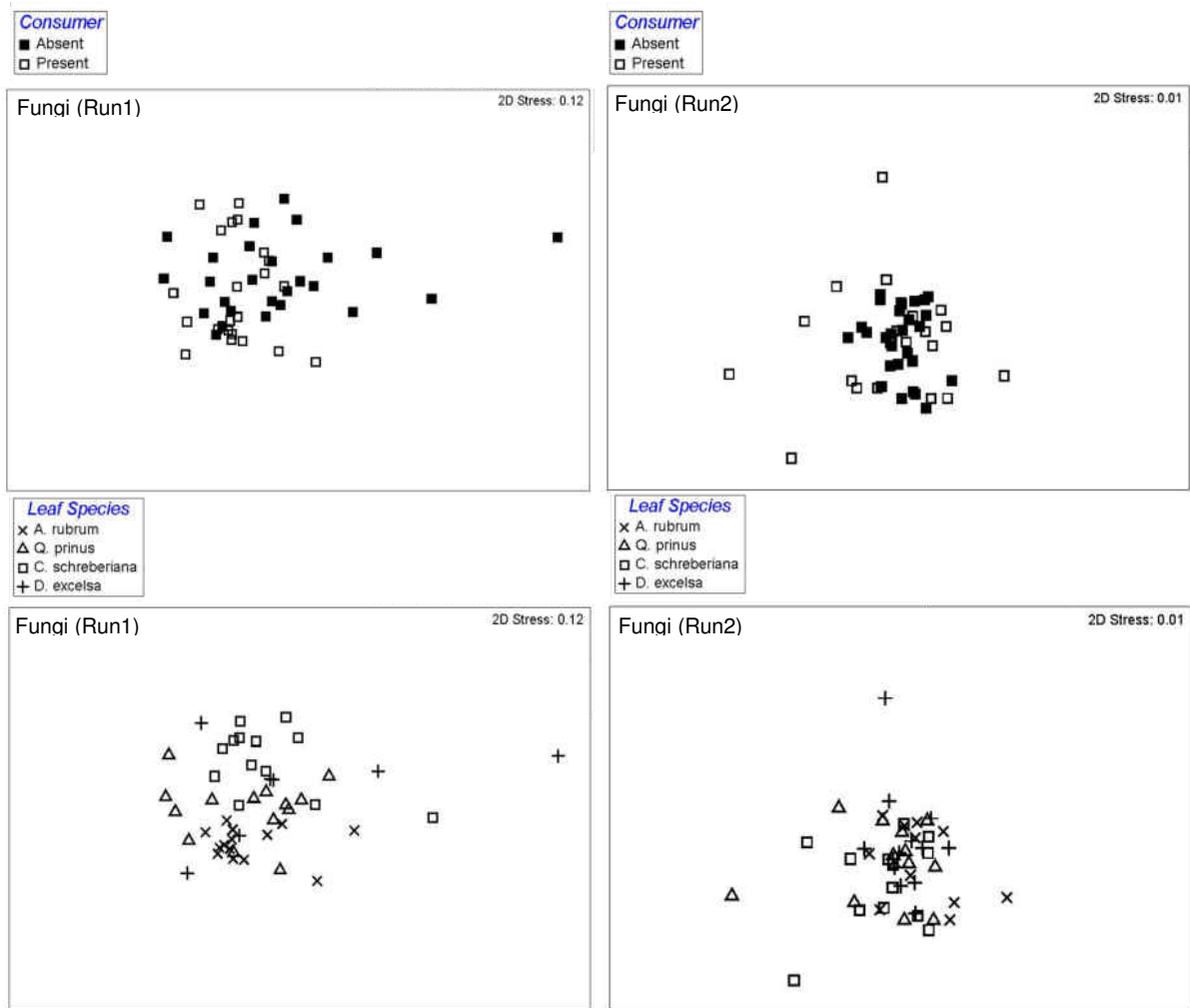
Figure 3.5. Non-metric multidimensional scaling (nMDS) ordination comparing fungal communities among two consumer treatments (absent and present) and four leaf types (*A. rubrum, Q. prinus, C. schreberiana, and D. excelsa*) for temperate laboratory trails (Run 1 and Run 2). Each point represents the square root of relative abundance of all TF peaks in a sample. In the top plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares. In the bottom plots, *A. rubrum* is represented by X's; *Q. prinus* is represented by triangles; *C. schreberiana* is represented by squares; and *D. excelsa* is represented by pluses.
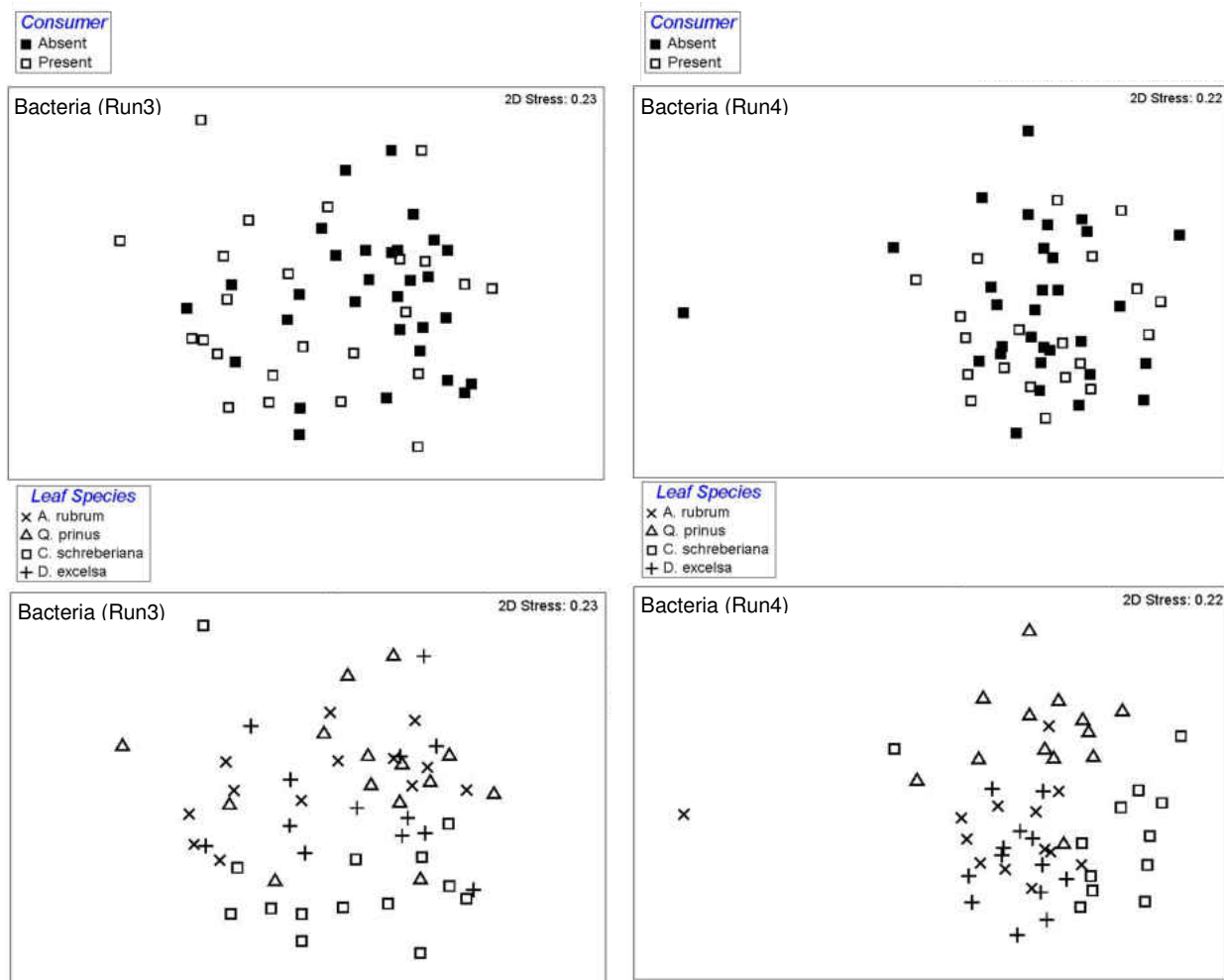
Figure 3.6. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial communities among two consumer treatments (absent and present) and four leaf types (A. *rubrum, Q. prinus, C. schreberiana, and D. excelsa*) for tropical laboratory trails (Run 3 and Run 4). Each point represents the square root of relative abundance of all TF peaks in a sample. In the top plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares. In the bottom plots, *A. rubrum* is represented by X; *Q. prinus* is represented by triangles; *C. schreberiana* is represented by squares; and *D. excelsa* is represented by pluses.
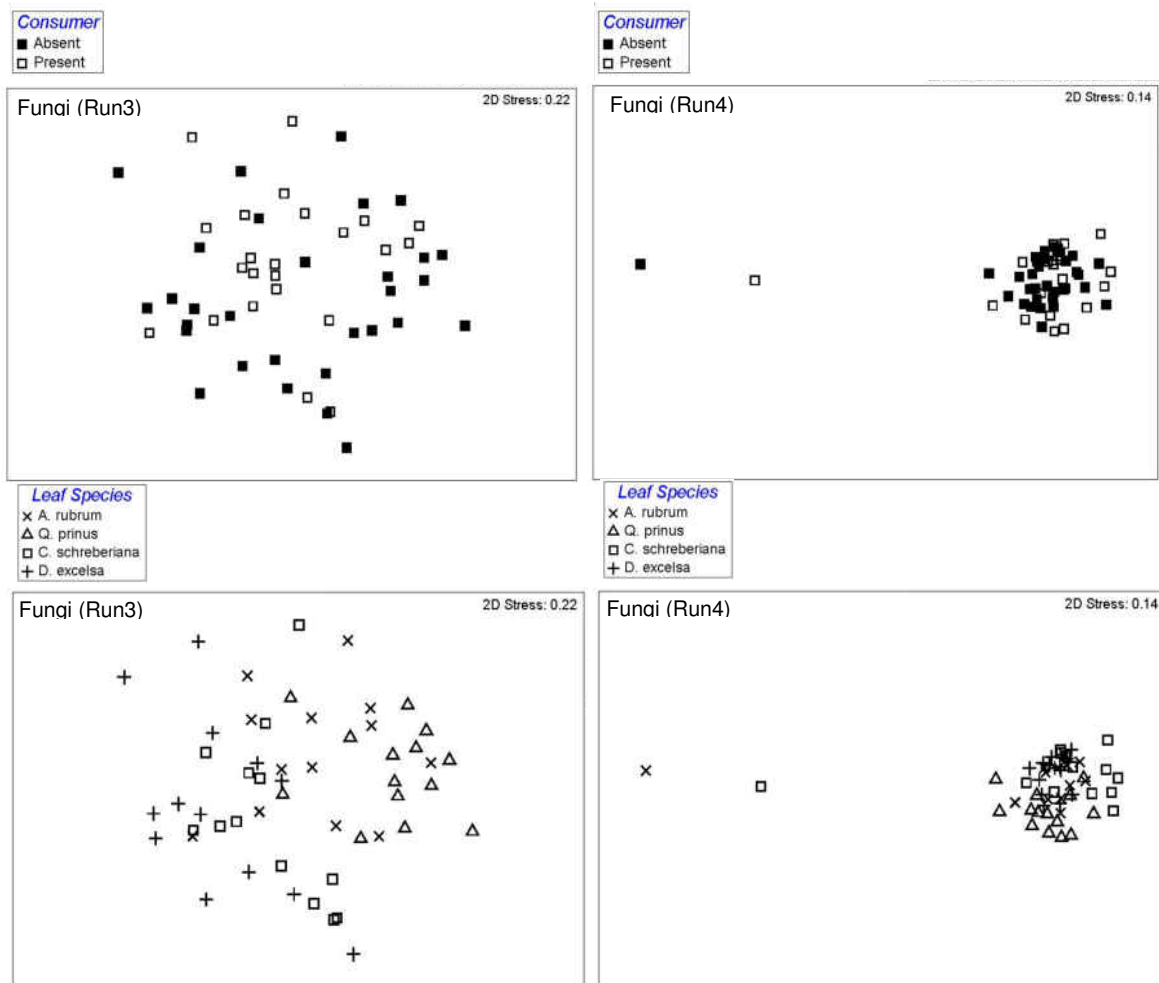
Figure 3.7. Non-metric multidimensional scaling (nMDS) ordination comparing fungal communities among two consumer treatments (absent and present) and four leaf types (*A. rubrum, Q. prinus, C. schreberiana, and D. excelsa*) for tropical laboratory trails (Run 3 and Run 4). Each point represents the square root of relative abundance of all TF peaks in a sample. In the top plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares. In the bottom plots, *A. rubrum* is represented by X; *Q. prinus* is represented by triangles; *C. schreberiana* is represented by squares; and *D. excelsa* is represented by pluses.

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Figure 3.8. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial communities among two consumer treatments (absent and present) for all leaf types in temperate laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares.

Figure 3.9. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial communities among four time intervals (7, 21, 35, 49 days) for all leaf types in temperate laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, day 7 is represented by squares, day 21 is represented by pluses; day 35 is represented by circles; and day 49 is represented by X.
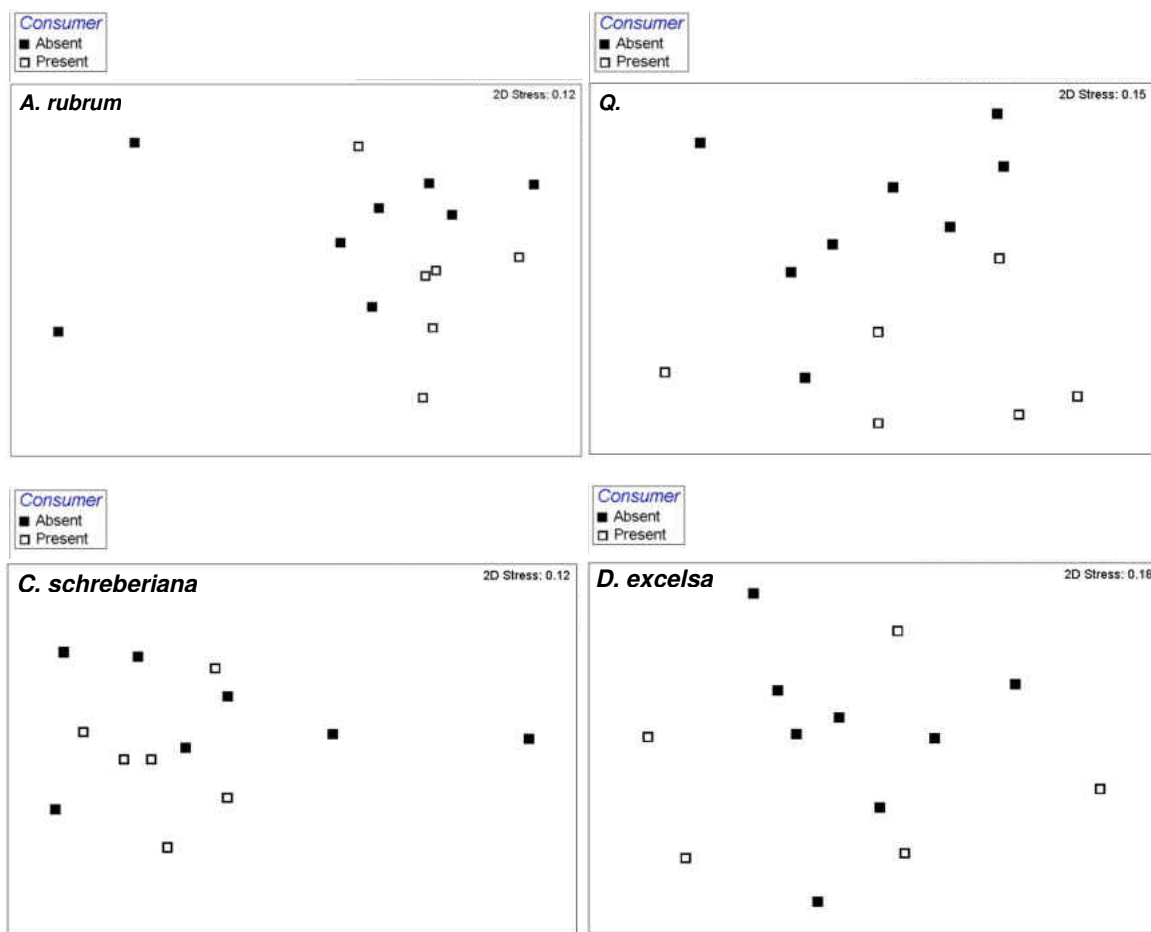
Figure 3.10. Non-metric multidimensional scaling (nMDS) ordination comparing fungal communities among two consumer treatments (absent and present) for all leaf types in temperate laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares.
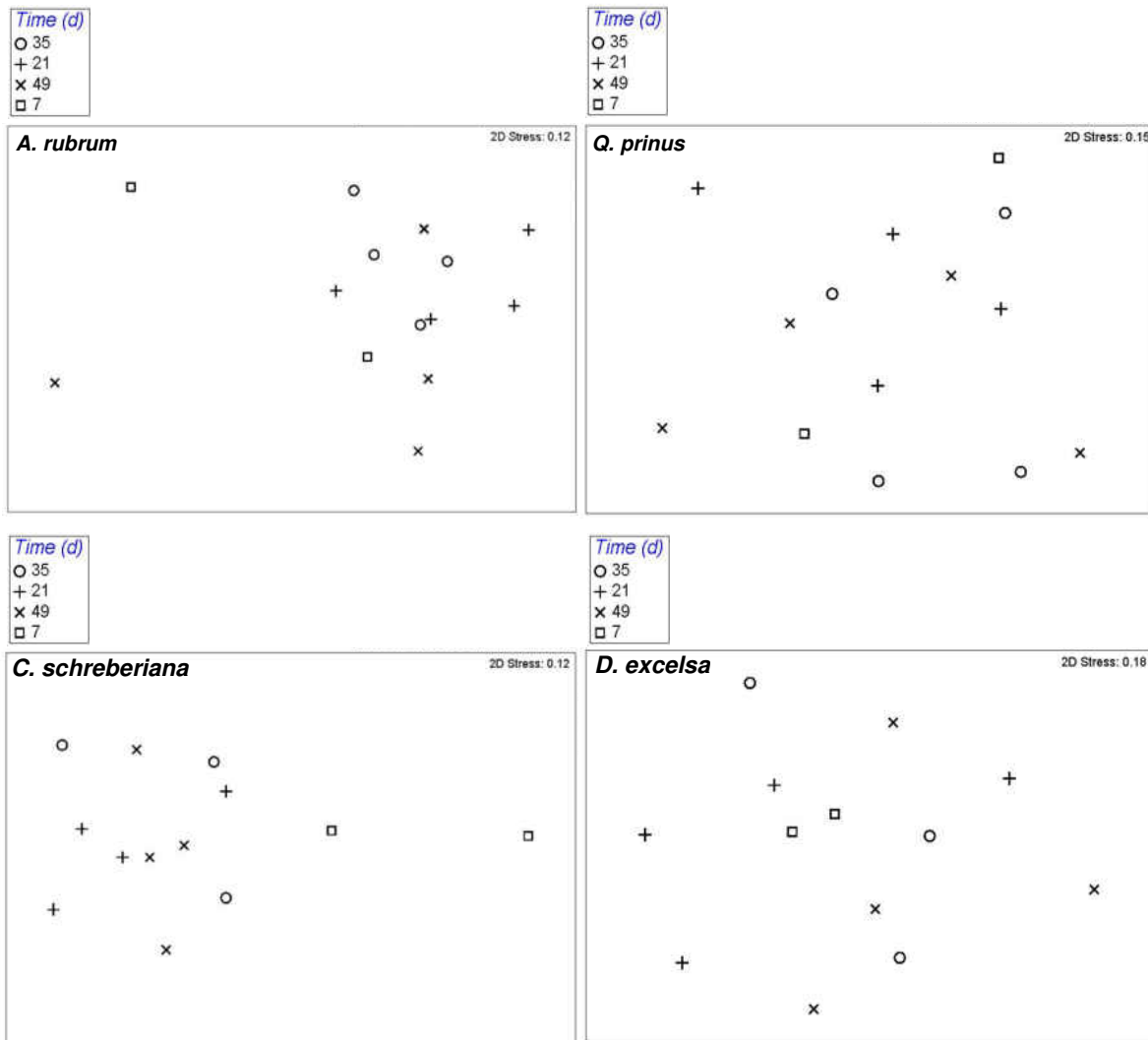
Figure 3.11. Non-metric multidimensional scaling (nMDS) ordination comparing fungal communities among four time intervals (7, 21, 35, 49 days) for all leaf types in temperate laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, day 7 is represented by squares, day 21 is represented by pluses; day 35 is represented by circles; and day 49 is represented by X.
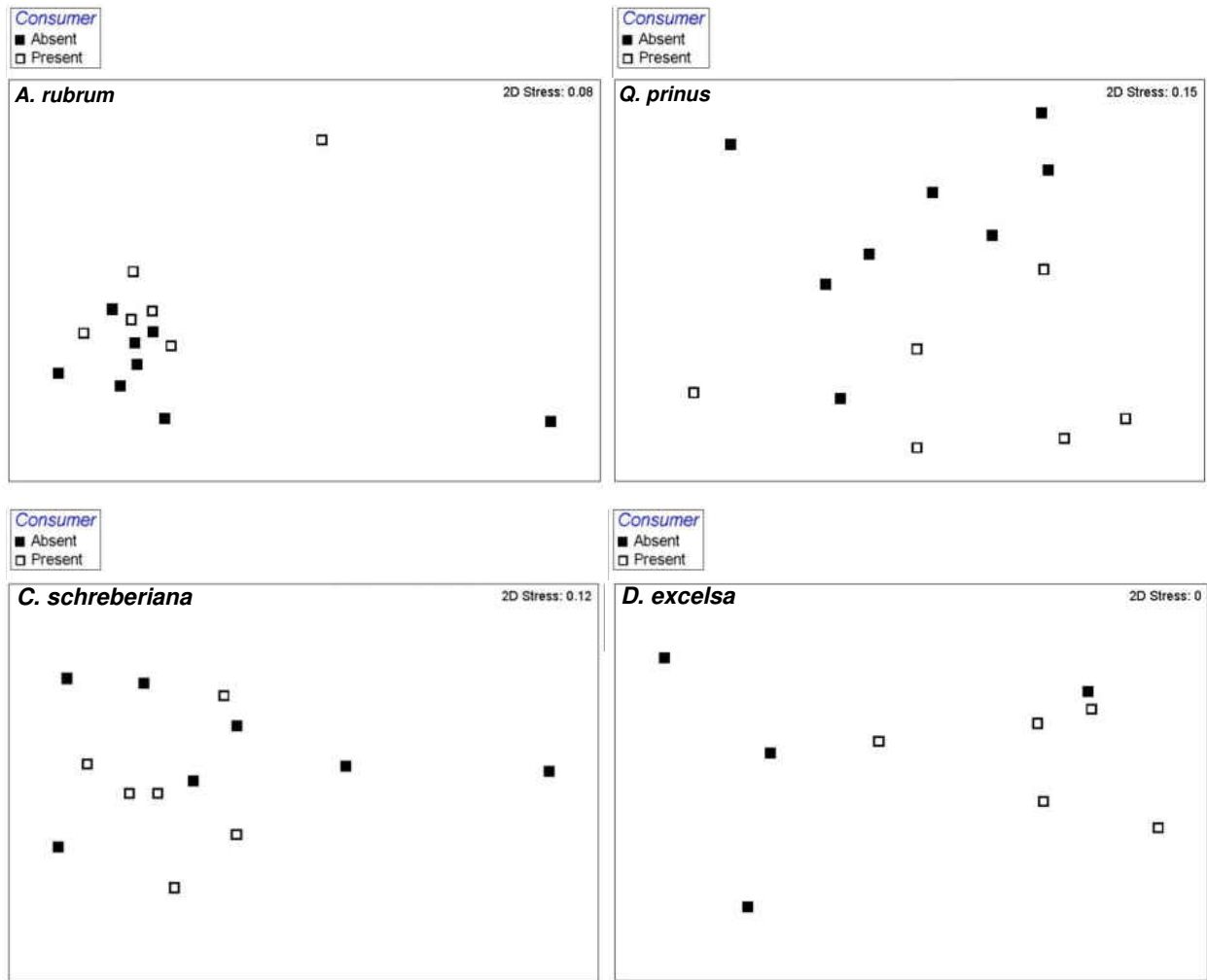
Figure 3.12. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial communities among two consumer treatments (absent and present) for all leaf types in tropical laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares.
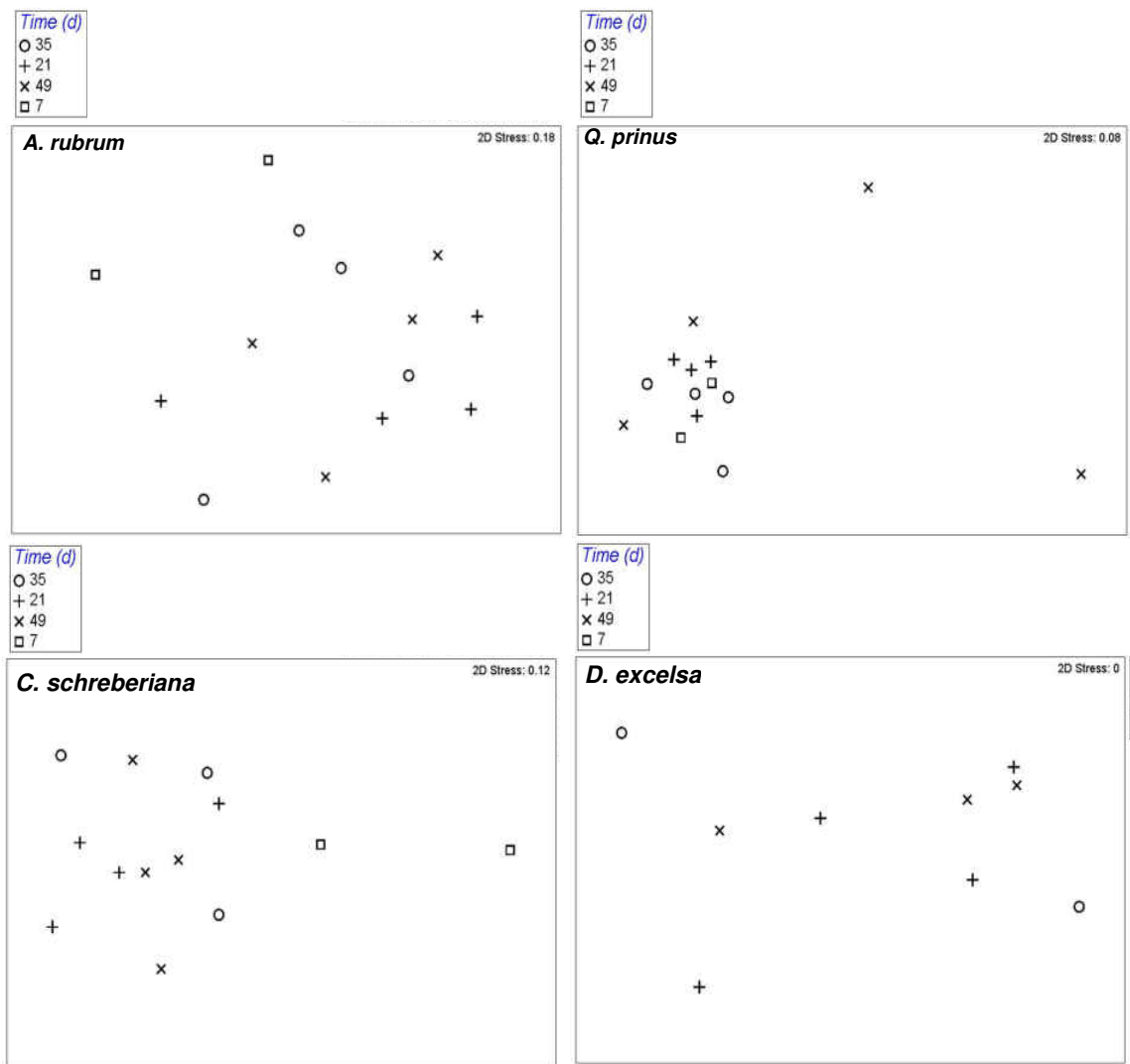
Figure 3.13. Non-metric multidimensional scaling (nMDS) ordination comparing bacterial communities among four time intervals (7, 21, 35, 49 days) for all leaf types in tropical laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, day 7 is represented by squares, day 21 is represented by pluses; day 35 is represented by circles; and day 49 is represented by X.
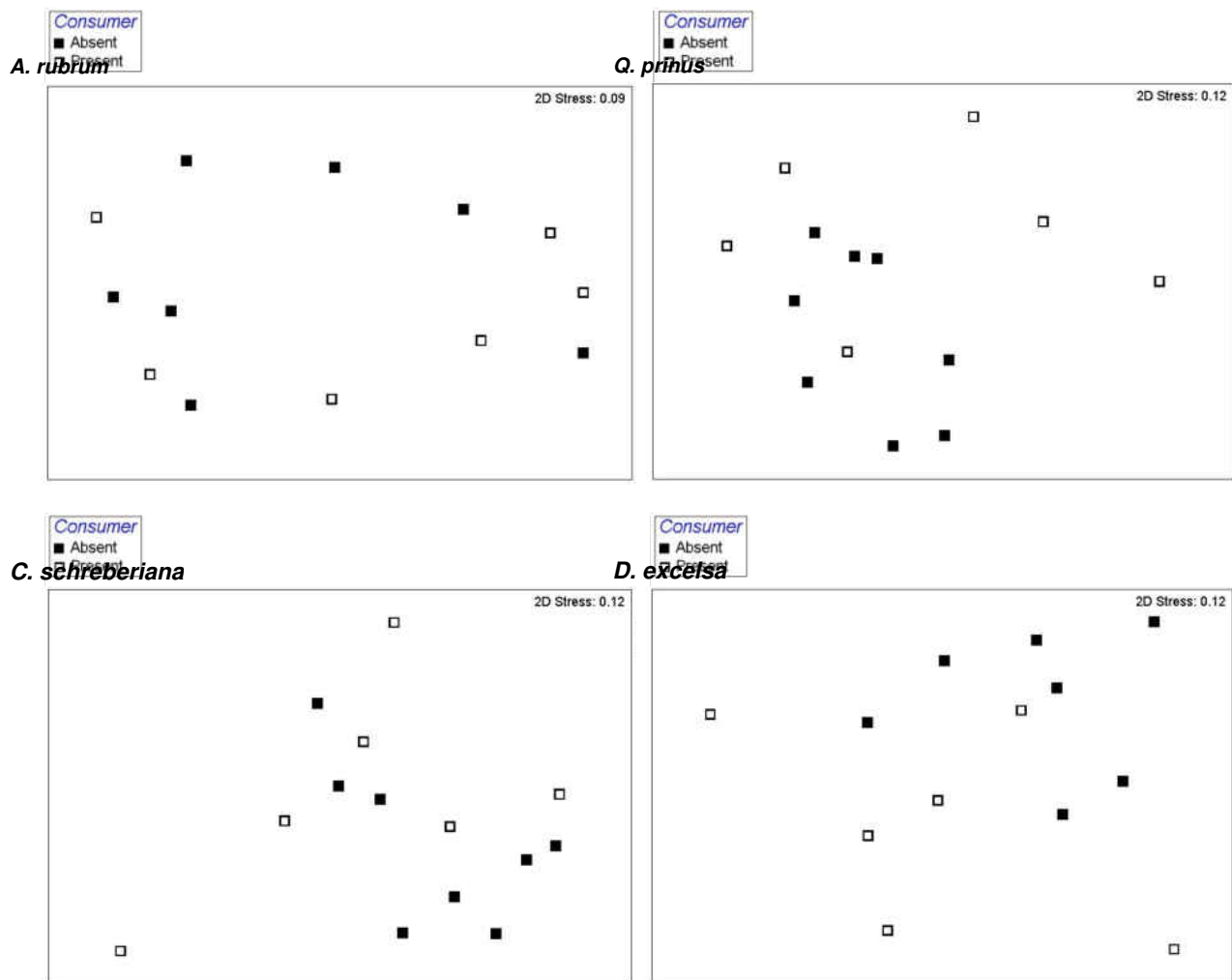
Figure 3.14. Non-metric multidimensional (nMDS) scaling ordination comparing fungal communities among two consumer treatments (absent and present) for all leaf types in tropical laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, replicates where consumers are absent are represented with filled squares and replicates where consumers are present are represented with open squares.
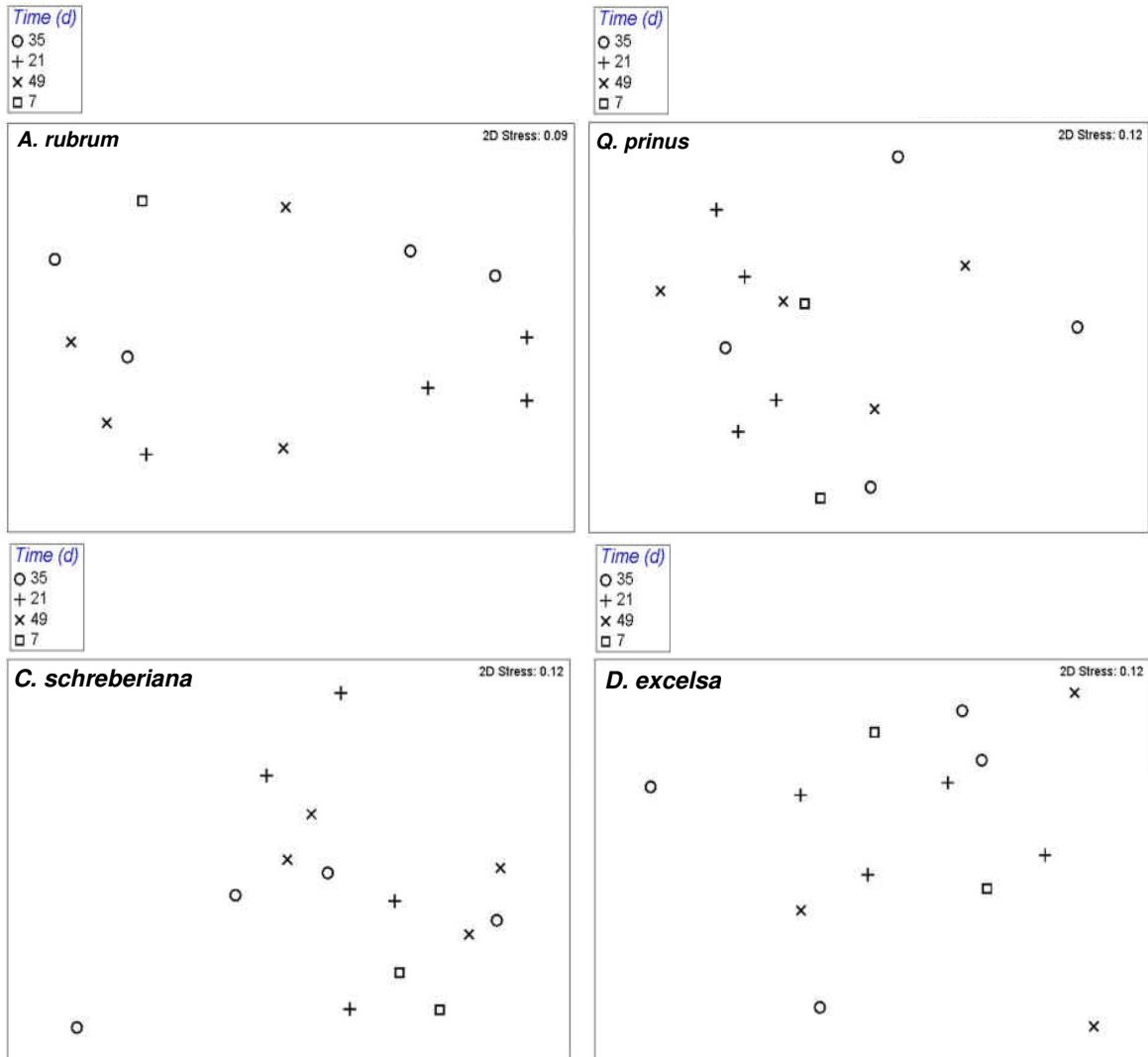
Figure 3.15. Non-metric multidimensional scaling (nMDS) ordination comparing fungal communities among four time intervals (7, 21, 35, 49 days) for all leaf types in tropical laboratory trials. Each point represents the square root of relative abundance of all TF peaks in a sample. In all plots, day 7 is represented by squares, day 21 is represented by pluses; day 35 is represented by circles; and day 49 is represented by X.
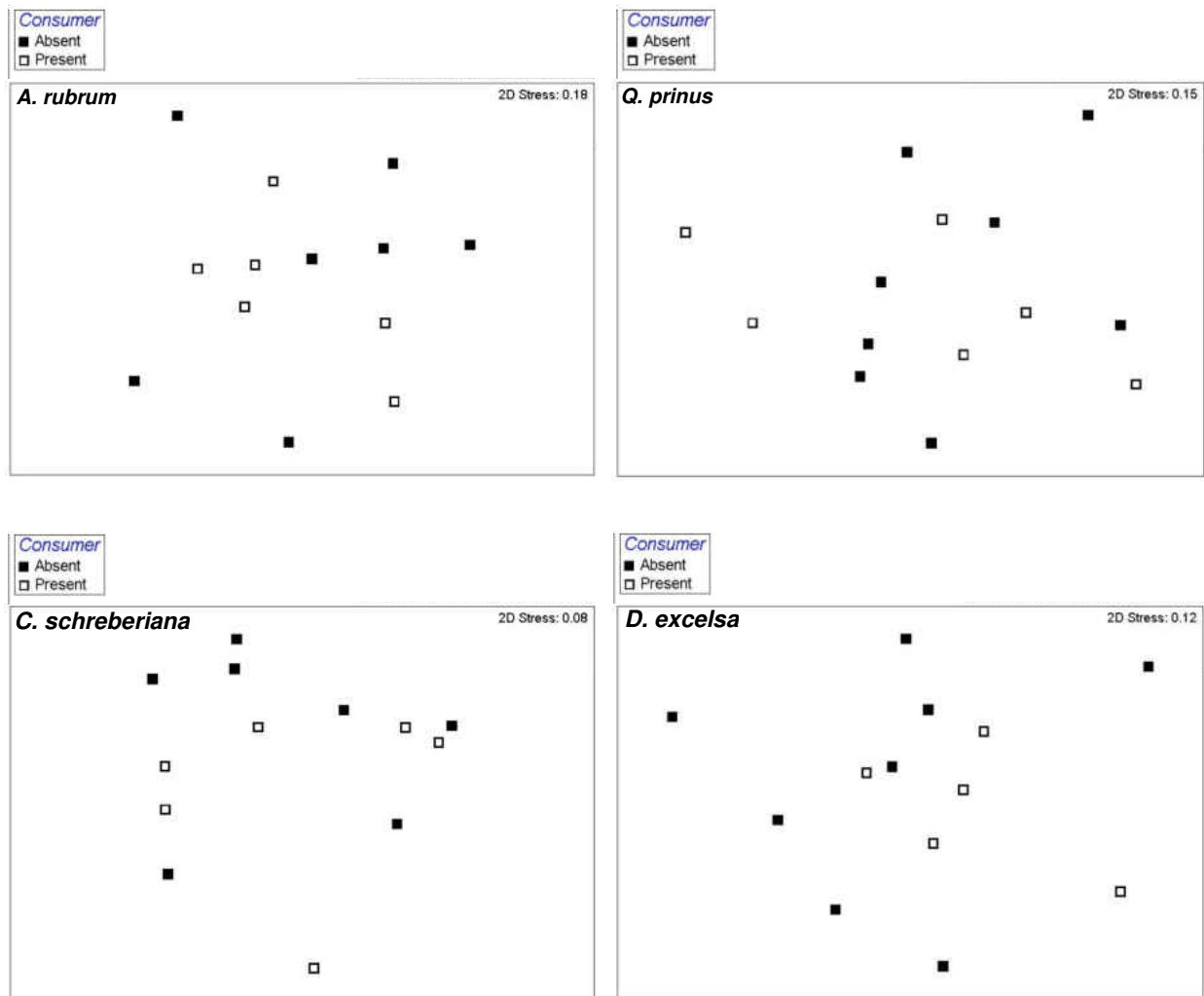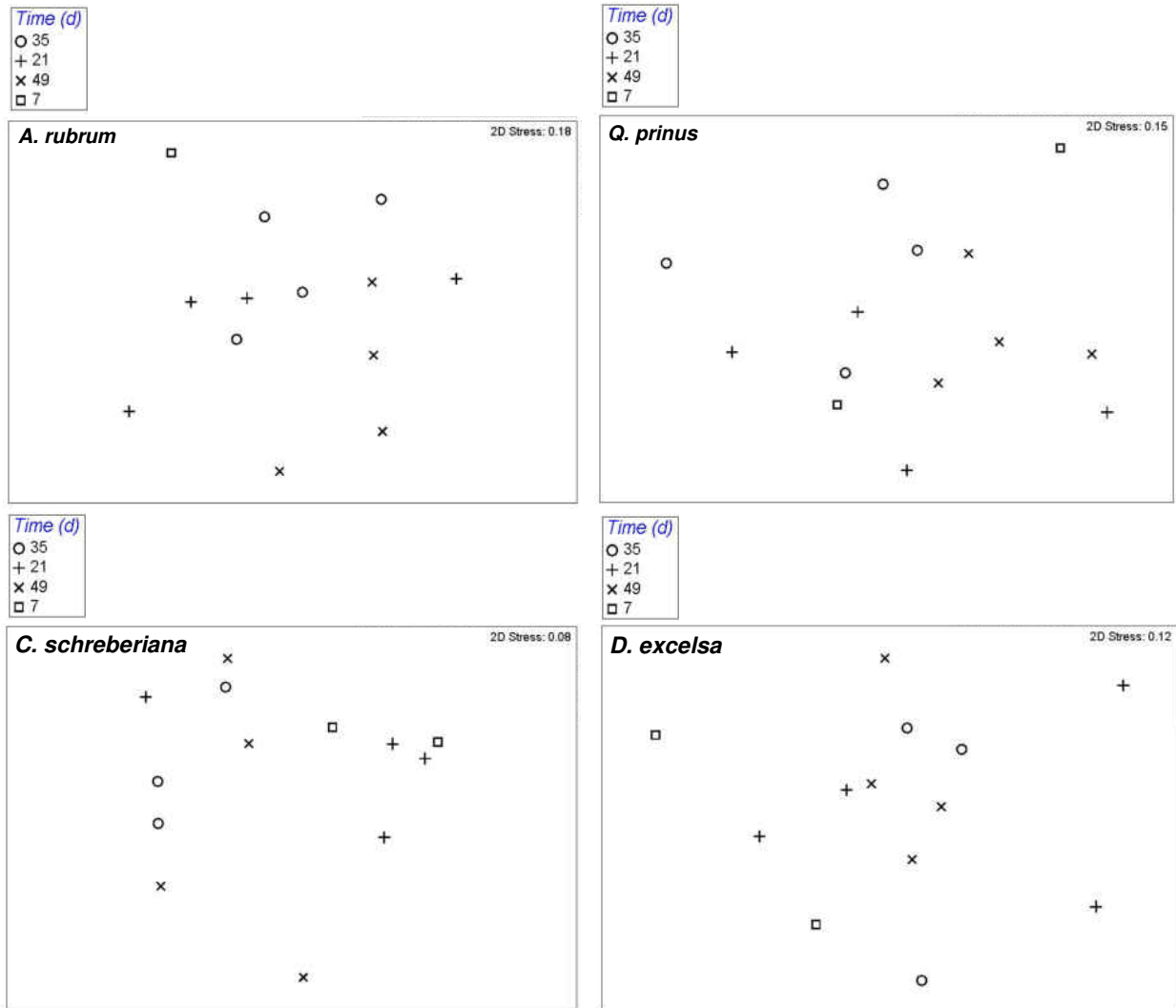
CONCLUSIONS

Organic matter processing in stream ecosystems has been studied in depth because it is an ecosystem function. In headwater streams, the breakdown of organic matter, such as leaves, fuels the stream with nutrients. Investigating microbial communities associated with leaves during the early stages of decomposition has provided a better understanding of the dynamics of colonizing consumers. Comparing these communities between different leaf types and regions gives insight into factors that can influence the ecosystem process.

In Chapter 2, I showed that region and leaf type influences the community composition of both bacteria and fungi. In addition, for all leaf types, there was significant difference in community over time. Although the trajectory of the succession of microbes is unclear, there is evidence that these microbial communities are changing throughout these early stages of decomposition. In Chapter 3, I identified important variables necessary for successful microbial mesocosm experiments. I was able to recreate weak trends that coincide with the findings of Chapter 2.

Community dynamics between bacteria and fungi during leaf decomposition adds to the continuing knowledge of this ecosystem function. Overall, I was unable to observe an influence on the community richness or Shannon's diversity on decay rates. This suggests that there may be a suite of microbes that function similarly and ultimately suggests that there is redundancy in these systems. In variable ecosystems, redundancy can be important as it can be indicative of the resilience of the ecosystem to respond to disturbances.

This study is unique in the fact that microbial communities have not been commonly assessed using molecular techniques between a temperate and a tropical region. Molecular

techniques are important because these techniques are capable of investigating community dynamics that would otherwise be very difficult to measure. Therefore, there is still a need for studies using molecular techniques in the field of stream ecology. Future studies may attempt to link microbial communities with their associated function within stream ecosystems by using a myriad of molecular techniques, such as next generation sequencing.

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