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Wetting and drying cycles and the fungal communities on leaf litter in streams

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WETTING AND DRYING CYCLES AND THE FUNGAL COMMUNITIES ON LEAF LITTER IN STREAMS

By

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ABSTRACT

Leaf litter is a major source of energy for streams in deciduous forests. Fungi play a critical role by converting the leaves into nutritional material for the rest of the food web. The breakdown of leaf litter and associated biota, including invertebrates and fungi, have been proposed as measures of stream health in systems affected by anthropogenic activity. Rates of leaf breakdown can be depressed in streams affected by acidity, metals, organic contaminants, and other stressors. Climate change may lead to alterations in stream hydrology such that streams experience more frequent floods as well as drying episodes. Fungal communities can be affected by the leaf litter they are growing on becoming emersed and re-immersed due to pulse-flow events. I examined the structure and function of fungal communities at several sites with varying emersion-immersion. I used DGGE in tandem with clone libraries to assess the community structure of fungi on leaves from the sites over a 15 week period. I also measured fungal biomass and microbial activity, which were closely related to each other throughout the sampling period. Sites that underwent an emersion-immersion cycle had lower activity than immersed sites initially, but sites had similar rates later after communities had become established. Overall, community composition and diversity varied among samples based on immersion, watershed, and time. Clone libraries revealed that the main taxa at my sites were not aquatic hyphomycetes, as most fungal studies have assumed, but terrestrial taxa.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1. BACKGROUND

In streams that run through deciduous forests, allochthonous organic matter inputs can account for over 99% of the annual energy budget (Fisher and Likens 1973). These fallen leaves are colonized by aquatic microbes, which begin the breakdown process (Kaushik and Hynes 1971). Aquatic fungi are the main microbial agent in the early stages of leaf breakdown (Suberkropp and Weyers 1996). Upon falling into the stream or being blown in, the leaf rapidly begins to leach polyphenols and carbohydrates (Suberkropp et al. 1976). This process can account for upwards of 25% of leaf mass depending on plant species. This leaching is usually done within a day or two. Within the first week fungi and some bacteria colonize the leaves and begin to condition them. Fungi use exoenzymes to break down polysaccharides (Jenkins and Suberkropp 1995) as well as using their hyphae to break into leaf tissue (Wright and Covich 2005). Both of these processes soften the leaf tissue, leading to easier abiotic fragmentation, release of fine particulate organic matter (FPOM), and increased palatability to shredding invertebrates. Fungal growth leads to a more nutritious food supply for invertebrates because they concentrate nutrients from the water column on the leaves. Multiple studies have shown that invertebrates feeding on leaves colonized by fungi have improved survival and growth (Canhoto and Graça 2008). Through these mechanisms, the aquatic fungi mediate the process of converting the leaf litter into a useable energy source for the rest of the stream food web.

Previous research has indicated that many anthropogenic stressors can affect the community structure, diversity, and ecosystem function of the aquatic fungi. Acidity, metal contamination, eutrophication, and other stressors have all had effects on leaf breakdown and associated fungi (reviewed in Gessner and Chauvet 2002; Krauss et al. 2011). Few studies have examined the effects of climate change on stream fungi and their function.

Climate change poses a large threat to the biodiversity and functioning of natural ecosystems in the coming century. All ecosystems are affected in some way by a changing climate. Stream ecosystems are affected by two main factors related to climate change: stream temperature and hydrology. Although rising temperatures can harm some stream biota such as trout, the effect of hydrological changes to the quantity of water as streamflow is probably a greater threat to most streams. Streams in the central United States, including Missouri, are especially susceptible to stress from reduced streamflow and possible stream drying (Covich et al. 1997). Furthermore, seasonal flow variability is expected to increase with larger peak flow events and more time with low or no flow in many streams (Mulholland et al. 1997). This greater range of flow causes leaf litter to be more likely to not spend its entire breakdown process continuously submerged but rather be exposed to air (emersed) during low flow and then re-submerged later (immersed) during higher flow.

Another aspect of stream drying is that decreased flow can lead to fragmented sections, where habitat is transformed from running water to isolated pools (Lake 2003). Many studies have examined the effects of flow variability on stream ecosystems overall (Lake 2000), and several have focused on leaf litter decay. However, all but a few have

focused on macroinvertebrate communities (Datry 2011b), with little attention to the fungal community (but see Maamri et al. 2001). The impact of this wetting, drying, and rewetting on fungal colonization, succession, and community structure and function on leaves in streams is the goal of this research.

1.2. OBJECTIVES

The goals of this research were (1) to compare the communities of fungi on leaves that were continuously submerged in flowing water, submerged in pools, or subject to emersion-immersion cycles, and (2) to determine if these differences in community structure are associated with changes in ecosystem function.

2. REVIEW OF LITERATURE

2.1 CLIMATE CHANGE AND MISSOURI STREAMS

Climate change will impact almost all natural ecosystems in extremely complex ways; our freshwater streams are no exception. Warmer temperatures lead to greater evapotransporation rates in watersheds, which can decrease the flow in streams. However, the increased surface temperature of oceans leads to greater rainfall on land. Overall, these two factors are estimated to yield a 5 - 20% increase in local stream discharge in the Missouri Ozarks (Figure 2.1, Milly 2005, 20C3M scenario). This increase in total discharge, however, is unlikely to be uniformly distributed across the year. A more likely scenario is that the precipitation will be focused through the fall and winter and a few major events during the spring and summer. This leads to an increased chance of flooding. The higher temperatures also lead to increased evapotranspiration, which increases the likelihood of dry spells in the stream channel during summer dry seasons (Mulholland et al. 1997).

This pattern is in line with the models of the Intergovernmental Panel on Climate Change (IPCC), which show a slight increase in extreme precipitation in the Ozarks region and a much larger number of dry days (IPCC Fourth Assessment Report: Climate Change 2007 Section 10.3.6.1). Some models specifically show an increase in severe storms and precipitation events for the southeastern United States (Section 14.3.1 working group II).

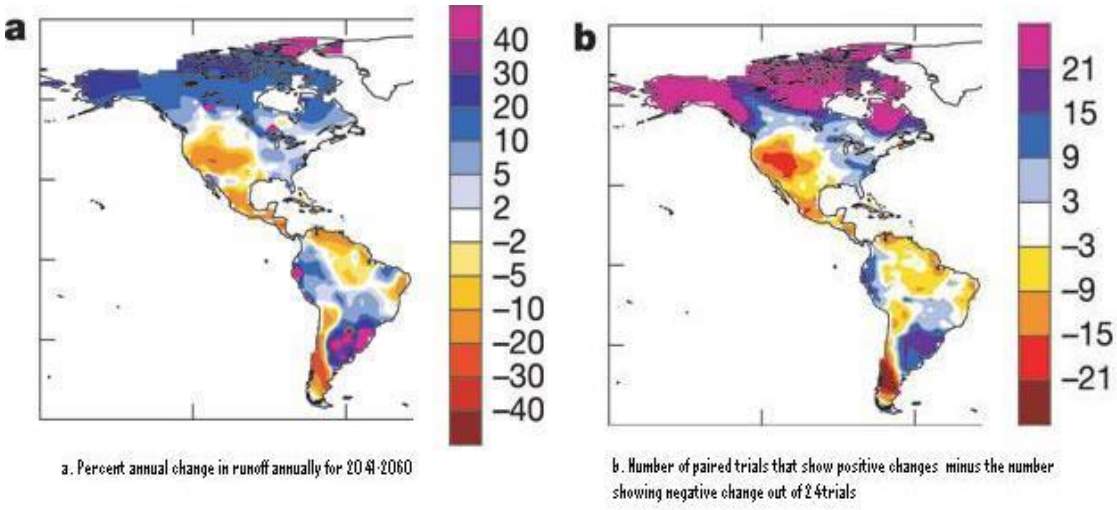


Figure 2.1. Projected change in annual runoff for the Americas. **(a)** The projected change in percent runoff. (**b)** The agreement among modeling runs for changes.

Increases in peak flows and decreases in average flow for the rest of the season are likely to impact the physical distribution of leaves in or around the stream channel. In a steadily flowing stream, leaf litter often accumulates along the banks and on the bottom of the channel in runs, and litter is often condensed in riffles where sticks and rocks trap the litter. With an increase in high flow events during winter, more of the litter will be carried off from these immersed positions and deposited on the stream bank. It will remain there until the next high water event, when it is likely to be re-submerged. Thus, a greater portion of litter is undergoing this pulsed immersion cycle rather than continuous immersion.

2.2 FUNGAL ECOLOGY OF STREAMS

2.2.1. Aquatic Fungus. Aquatic hyphomycetes have long been thought to be the dominant players in leaf litter decay in streams (Bärlocher 1992, Suberkropp 1992). They are a polyphyletic group with multiple origins (Belliveau and Bärlocher 2005); they

sporulate in water, and produce iconic multiradiate or sigmoid spores, which are often coated in sticky mucilage thought to enhance their ability to attach to leaf litter (Read et al. 1992). Their detection and identification has been traditionally done with microscopy by visually counting spores from the water or from incubated leaves. The spores used for identification have adapted in a state of convergent evolution making identification somewhat ambiguous. (Bärlocher 2007).

Aquatic hyphomycetes are not the only species of fungus present in the stream. While they have received the bulk of attention, other non-spore producing fungi, even those associated with terrestrial conditions, are often found in the streams (Bärlocher and Kendrick 1974), but their functional contribution has been largely overlooked. Several recent studies using molecular methods to describe stream fungi, including clone libraries, have revealed that taxa other than aquatic hyphomycetes are common in streams and may be abundant (Seena et al. 2009, Kelly et al. 2010).

2.2.2. What Are They Doing? Leaves falling into streams in autumn compose a major energy source for stream communities (Allen 1995). The presence of stream fungi was noted but largely ignored until Kaushik and Hynes (1971) demonstrated the role they played in breaking down and conditioning leaf litter for invertebrate consumption. Kaushik and Hynes (1968) had observed that the protein content of leaf litter goes up over time as the leaves sit in the stream. They used antibacterial and antifungal compounds to show that the protein was indeed from fungi rather than bacteria. Studies have subsequently shown that colonization by fungi improves the growth and survival rates of the macroinvertebrates feeding on leaves compared to sterile leaves or leaves colonized solely with bacteria (Canhoto and Graça 2008). Ergosterol, which acts in

fungus in a similar role that cholesterol does in mammals, can be used as a proxy for fungal biomass (Newell and Fallon 1991). This has shown that 18-23% of the mass of leaves decaying in a stream can be attributed to the fungus itself. (Gessner 1997, Gessner et al. 2003). Additionally, the fungi are considerably more active than their bacterial counterparts, with 95% of the microbial community attributable to fungi (Findlay and Arsuffi 1989, Suberkropp 1997). In addition to conditioning the leaves for shredding invertebrates, the spores released by the aquatic hyphomycetes can be consumed by filter feeding macroinvertebrates (Bärlocher and Brendelberger 2004), and the leaf degradation by fungal exoenzymes can generate FPOM, which is released into the stream channel (Suberkropp and Klug 1980).

This litter processing, then, is assumed to be the primary functional role of aquatic fungi in streams. As such, it has been proposed as a metric for measuring functional integrity of streams (Gessner and Chauvet 2002). However, the fungi do play other secondary roles in the environment such as metal sequestration and degradation of xenobiotics (reviewed in Krauss et al. 2011).

2.2.3 Linking Fungal Structure and Function. The link between biodiversity and functioning of biological communities has been a major research focus in the last two decades (Hooper et al. 2012). Several recent studies have used experimental approaches to look at diversity-function relationships in aquatic hyphomycetes. Bärlocher and Corkum (2003) found significant effects of species richness and identity, but this effect was much smaller than positive effects from nutrients. Treton et al. (2004) reported enhanced breakdown of alder leaves from a combination of two species compared to either species alone. In the most comprehensive study published to date, Dang et al.

(2005) found no significant effect of diversity on leaf breakdown in experimental microcosms, although they did find reduced variability with more species present. Changes in community structure do not always lead to changes in function; even loss of diversity does not always imply a loss of function due to functional redundancy within the community.

2.3 STREAM FUNGI UNDER CLIMATE CHANGE

Many studies have looked at a wide variety of factors which can impact structure, diversity, or function of stream fungal communities. Metals, acidity, eutrophication, and xenobiotics have been commonly studied (reviewed in Gessner and Chauvet 2002, Krauss et al. 2011). For example, Niyogi et al. (2002) found lower diversity in streams affected by mine drainage in Colorado, and pH and zinc were related to the loss of diversity. Furthermore, Niyogi et al. (2009) also found reduced diversity at sites affected by mine drainage using molecular methods.

Surprisingly, few studies have examined the effects of climate change on stream fungi and their role in leaf breakdown. Ingold (1975) proposed that the functional role of aquatic hyphomycetes would have the most impact in cool, clean, well-aerated streams in deciduous forests. As the environment moves away from these ideal conditions the key functional role of aquatic hyphomycetes may begin to fall off as other species take over.

Climate change will impact aquatic fungal communities in several ways. It will increase water temperature, which will directly impact the life cycle of the aquatic fungi (Bärlocher et al. 2008). Rising atmospheric CO_2 will change the chemistry of leaves to a

more carbon-rich, nutrient-poor ratio (Tjoelker 1999). All of these effects are likely to influence the fungal community structure in streams slowly over time.

Drying events and drought do not exert this same slow steady selective pressure on the community but rather act intermittently and immediately. These drying events are likely to decrease diversity which could lower ecological functioning, especially when other stressors are present, such as eutrophication or increased concentrations of metals (Pascoal et al. 2009). These rapid changes in environmental conditions are also likely to knock out some keystone species that may not be as adapted to the new environments.

Several studies have looked at how drying periods affect the macroinvertebrate community of streams (reviewed by Lake 2000) and subsequent function of leaf litter breakdown rates (Pinna et al. 2004, Acuña et al. 2005, reviewed by Datry et al. 2011b). However, few have looked at drying effects on the fungal community. Maamri (2001) is the only study I am aware of to date that has attempted this, and it was limited to conidia identification for community assessment. In the case of drying versus wetting conditions, conidia are most likely not the best candidate for approaching the question due to the very nature of conidia analysis, which involves incubation in water (Bärlocher 2005).

The fungal community's role in litter processing cannot be overlooked. Mass loss due to microbial processing is often more important than processing due to macroinvertebrates. This is especially the case when the litter is emersed (exposed to air) or in streams where macroinvertebrates are less common such as in the Great Plains. (Hill 1992, Corti et al. 2011). In a study by Datry et al. (2011a), microbial activity did not differ enough to significantly explain differences in litter breakdown rates when

comparing temporary streams that had been impacted by drought the previous season. In these temporary streams, the major impact was on the invertebrate assemblages.

A large portion of leaf litter breakdown occurs in the winter and spring when water supplies may not be as limited. This could ameliorate the impact of drying events to some degree, but perennial streams that go completely dry tend to have a lag effect in leaf breakdown rates the following year. Whether or not this is only due to the impact of the drying on the invertebrate community, or some combination of invertebrates and fungi, is still unclear (Maamri et al. 2001).

2.4. REVIEW OF METHODS FOR FUNGAL COMMUNITIES

There are two general approaches today for the study of aquatic fungi on decomposing leaves. The traditional approach has been the examination of conidia from aquatic hyphomycetes (assumed to be dominant stream fungi) on leaves by light microscopy using identification guides (Gulis et al. 2005). Modern methods involve molecular analyses of stream fungi, and include DGGE, T-RFLP, ARISA, clone libraries, and other techniques. This review and my research focus on the use of DGGE and clone libraries.

2.4.1. The Value of Denaturing Gradient Gel Electrophoresis (DGGE). The traditional method to identify stream fungi relied on the collection and identification of conidia, which are asexual spores. There are two major problems in using conidia to identify fungal taxa. The first is that some taxa do not produce conidia. The second, is that conditions for conidial growth and release are not always met while hyphal growth does continue. DGGE often reveals more phylotype diversity than traditional

identification of conidia (Nikolcheva et al. 2003, Raviraja et al. 2005). Beyond this, simply using conidia to establish who is a key player might be a limited approach as previous studies have found that leaf decomposition extends to species outside of aquatic hyphomycetes (Nikolcheva and Bärlocher 2004, Seena et al. 2008). DGGE analysis has shown that the presence of non-aquatic hyphomycete fungi may become more important under stressful conditions (Chergui and Pattee 1988, Raviraja et al. 1996). Furthermore, even if aquatic hyphomycetes are present, their conidia production can be limited by stressors (Abel and Bärlocher 1984, Niyogi et al. 2009).

2.4.2. How DGGE Works. The basic flow of analyzing an environmental community with DGGE involves the following: 1) extracting DNA from the environmental sample (often done with a DNA extraction kit), 2) amplifying the DNA by PCR, and 3) running the PCR product by DGGE to separate the environmental amplicons.

The PCR primer selection has a large impact on the resulting DGGE as it determines what species are qualified as fungus. ITS3GC(F) and ITS4(R) are a pair used commonly for fungal analysis. This pair covers the internal transcribed spacer region 2 (ITS2) of the rDNA gene. The ITS2 region is chosen because of its semi-conserved nature in fungus. ITS3GC and ITS4 are considered to be a "Universal Primer" pair for fungi (White et al. 1990). They appear to have fairly robust discrimination potential for aquatic fungi separating 9 out of 10 species of aquatic hyphomycetes including two strains of one species (Seena et al. 2010a).

Amplicons from PCR will be approximately the same length but will vary slightly in their sequence. Because of the triple hydrogen bonding structure between G:C and

double bonds in A:T (Watson and Crick 1953), a slight change in sequence will mean the double strands will melt at different temperature or concentrations of a chemical denaturant. The latter is exploited for the purpose of DGGE. The gel is made from a matrix of polyacrylamide with an increasing concentration of denaturant added to create a linear gradient of chemical instability for dsDNA. (Muyzer et al. 1993). The DNA will migrate into increasing denaturant concentrations and eventually "melt". However, if the strand completely melts into two single strands, both will continue to migrate all of the way out of the gel. A GC clamp is added to the PCR primers to prevent this from happening. The clamp is a ~40bp string of all guanines/cytosines giving it a very high effective melting temperature (Muyzer et al. 1993).

2.4.3. Weaknesses of DGGE. The first major pitfall of DGGE, and most other molecular methods, is that it does not matter if the DNA is from metabolically active fungus. There is no guarantee that the bands are not resulting from dead or dormant microbes. DNA in dead cells is thought to degrade rapidly but can stick to clay and other particles to extend its life in the environment (Blum et al. 1997). By taking a series of samples over time, however, we can safely assume that at some point the DNA would be degraded. Another difficulty with analyzing a DGGE is that a single band could represent more than a single species if they both species' DNA shares a similar GC content and therefore similar mobility in the gel (Muyzer et al. 2004).

Because of these limitations, it is difficult to use DGGE to assess total species richness. More commonly, it is used to compare relative abundance of the major taxa in a community. An individual band should be considered a phylotype or operational

taxonomic unit (OTU) rather than an individual species. However, by using tandem clone libraries, several of these pitfalls can be worked around.

2.4.4. Clone Libraries. To date, three studies have addressed fungal stream communities using clone libraries: Kelly et al. 2010, Harrop et al. 2009, and Seena et al. 2008. The approach used in each case is that of a "shot gun" approach, in which clones are created en mass, from an environmental DNA extraction source, and then sequenced. The primary value for this "shot gun" method is to see what species are present. Additionally, one can divide the number of clones with the same sequence, by the total number of clones, to determine relative abundance. This approach re-sequences the more common species repeatedly and sometimes fails to capture important, but less abundant, species. Each of the recent studies found a significant portion of the community was either unidentifiable, or not an aquatic hyphomycete. By using a clone library in tandem with DGGE, it is possible to identify specific bands, and see their relative importance within the community, using band intensity as a proxy for percent of community.

3. MATERIALS AND METHODS

3.1. DESCRIPTION OF SITES

Six streams for this study were situated in Phelps County, Missouri. Three sites (AYS, YBPL, and KT) were in the Mill Creek drainage basin near the town of Newburg. Three other sites, TT, BB, and Quar, were all located slightly north or east of Rolla. All sites had two sub-sites, one "wet" site immersed in the stream and one "dry" site on the bank, with the exception of the AYS sites, which were located in isolated pools along Mill Creek. All sites were located in relatively pristine forested catchments, with limited human activities in the immediate vicinity of the sites.

Yelton Spring is a karst-fed spring located 20.7 km S.W. of Rolla (31.2 km driving) on County Road 7630. It represents the perennial headwaters for Mill Creek. There is another branch of Mill Creek that is ephemeral that continues into higher reaches of the catchment. The first sample site was located on this intermittent branch above Yelton Spring (AYS) (37.817065, -91.939409). The second sample site is located 2.4 km downstream from AYS, and is accessible from Forest Service road 1581, (37.837445, -91.939344). The third site is located on a tributary of Mill Creek called Kaintuck Creek (KT); it is accessible from Country Road 717, (37.869992, -91.928036).

Tanager Trails Ozark Nature Reserve, owned by the Audubon Society, is located just north of Rolla, accessible at the end of Meriweather Ct. Tanager Trails (TT) stream is a small, first order stream (37.963490, -91.778423). It is an intermittent/perennial stream depending on the year; some flow comes from a small spring.

The final two sites were both on unnamed low order streams to the east of Rolla. The first, accessible from Co Rd 3040, is in a stand of cedar trees below a quarry turned into a small lake. The water source for this intermittent stream is likely ground flow from the quarry: site Quar (37.966431, -91.723885). The other site, along Highway BB, is a second order, spring fed stream: site BB (37.951689, -91.688998).

3.2. WATER CHEMISTRY

Temperature, conductivity, and dissolved oxygen were all measured using a YSI 85 multimeter. Soluble reactive phosphorous (SRP) and dissolved inorganic nitrogen (DIN) were determined from single measurements taken from fall of 2011 through spring 2012. Standard methods (APHA 2007) were used for the measurement of these nutrients.

3.3. LITTER INCUBATION AND PROCESSING

Maple leaves were collected just after abscission, autoclaved, and cored. Twenty 1.8 cm maple (*Acer saccharum*) cores were placed in 1x1mm grid size mesh bags. Twelve litter bags were placed at each site, six submerged, and six at the water line. The groups were tethered to bank vegetation. Two litter bags were harvested from each site, one submerged and one on the stream bank, after being incubating for 21, 62, 83, and 104 days. Harvested litter bags were stored on ice until processing in the lab. Cores were gentle rinsed with ultrapure water to remove sediments and macroinvertebrates. Cores were used immediately for the respiration assay or stored at -20^{o}C for DNA extraction. Cores from the respiration assay were stored in methanol (at -20^{o}C) for ergosterol measurement.

Respiration was measured following protocols described by Niyogi et al. (2001). Three cores from each site were added to sealable 27 ml vials filled with stream water from the YBPL site in a series. Each site was tested in triplicate. Controls containing only stream water were tested in between each site set. The vials were incubated at room temperature for 6 hours, at which point dissolved oxygen was measured using a YSI 85 meter. The difference between the control vials and those with leaves from the sites was used to determined respiration by microbes on the leaves.

Ergosterol was extracted by high performance liquid chromatography (HPLC) using a modified version of the protocol used by Suberkropp and Weyers (1996). Tubes were filled to 8 ml with methanol and incubated for 2 h at 80^{o}C, with periodic shaking. 1.5 ml of 4% KOH in methanol was added, and tubes were refluxed for an additional 30 min. Tubes were decanted into clean tubes. Ergosterol was then partitioned into pentane using a series of separations. The pentane was allowed to evaporate and the resulting residue was dissolved in 1 ml of HPLC grade methanol, and filtered with a 0.45 µm filter. Samples were run on a C_{18} column on a UV600/P600 HPLC (LabTech, Colombia, MO, USA) and eluted with methanol at 2 ml min $^{-1}$ at 20^{o}C (peak elution time of 10.5 min). Peak areas were compared with external standards to estimate concentrations. Ergosterol values were converted into fungal biomass assuming a conversion factor of 5.5 mg ergostorol /g fungi (Gessner and Chauvet 1993).

3.4. FUNGAL COMMUNITIES

3.4.1. DNA Extraction and PCR. DNA was extracted from 3 leaf cores using an environmental DNA extraction kit (MoBio Ultraclean Soil DNA kit, Carlsbad,

California, United States) as per manufacturer's instructions. PCR was carried out using the primers: ITS3GC (5′-CGCCCG CCGCGC CCCGCG CCCGGC CCGCCG CCCCCG CCCC GCATCG ATGAAG AACGCA GC-3′) and ITS4 (5′-TCCTCC GCTTAT TGATAT GC-3′) (White et al. 1990, Muyzer et al. 1993) and PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire UK). PCR cycles were set as described in Nikolcheva and Bärlocher (2005). All PCR products and extracted DNA were stored at -20 °C until used. PCR was carried out on a PTC-200 thermocycler (BioRad Laboratories, Hercules, CA, USA). The target gene amplified by this reaction is ~370bp long.

3.4.2. DGGE Specifications. PCR products from the fungal ITS primers were analyzed by denaturing gradient gel electrophoresis (DGGE). The DGGE protocol (Jackson et al. 2001, Niyogi et al. 2009) used a D-Code System (Bio-Rad, Hercules, CA) on polyacrylamide gels using a 30 to 70% denaturant gradient (100% denaturant has 7 M urea and 40 % formamide). The gels also contained a gradient of polyacrylamide from 8 to 12 % (w/v). Electrophoresis was carried out in 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer at 55V for 16 hours at 60 °C. Gels were stained with SYBR Green for 30 min and visualized on a UV transilluminator.

3.4.3. Clone Libraries. Competent cells were created using a modified version of Inoue et al. 1990. *E.coli* was grown overnight at 18^{O}C in SOB (Super Optimal Broth). The culture was then chilled on ice for 10 min, spun down at 4000rpm at 4^{O}C for 10min, and resuspended in 8ml TB (Terrific Broth). Cells were chilled and spun a second time but resuspended in only 2ml TB the second time. 140µl DMSO was added, and cultures were chilled on ice an additional 10min. Cells were aliquoted into Eppendorf tubes and frozen in liquid nitrogen, then stored at -70^{O}C until use.

Ligation was carried out with a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. 5ul of the ligation product were added to the competent cells and incubated on ice for 30 min. Cells were then heat shocked for 1 min in a 42^{o}C water bath, and returned to the ice. 250 µl of SOC (SOB with glucose) was added to each tube. Tubes were shaken in a 37^{o}C incubator for 1hour. Contents were plated on to TB with ampicillin and incubated overnight. Plates were refrigerated at 4^{o}C until use. White colonies were selected at random for PCR. PCR was set up using growing colonies directly as the template by touching the pipette tip to the colony and then pipetting up and down with the PCR reaction mixture.

Each clone's PCR product was run in DGGE as above alone with a lane containing the environmental PCR product and a mixture of all clones run in each gel for comparison purposes. Bands in the mixed lane that matched bands in the Environmental lane were selected for sequencing. It is important to note that my clone library was not a "shotgun sweep" of what species were present, but a focused library based on matching bands from the environmental DGGEs. This means that the relative count of a species in the library is not directly proportional to how common a species was in the environment.

The entire ITS region has been suggested for barcoding (Rossman 2007). Our limited section (primers ITS3 and ITS4 (White 1990)) of this region gives us poor resolution to differentiate species. However, in most cases genus level identification was possible.

For sequencing, all samples were reamplified with PCR from the original PCR product and cleaned using QIAquick PCR purification kits (QIAGEN, Hilden, Germany). ~100 ng was run for sequencing, BigDye Xterminator purification kit was used for the

sequencing reaction. Sequencing was run on a Applied Biosystems 3130 Genetic Analyzer using Sequence Analysis software 5.2 (Applied Biosystems).

Contigs were generated using forward and reverse primer reads (ITS3GC and ITS4) using CAP3 software (Huang and Madan 1999). BLAST searches from the NCBI database were run using "Nucleotide Collection (nr/nt), highly similar sequences (megablast)". Several of the species had an uncultured "Ascomycota species" as the first hit. If there was a hit with a known genus in the search results, the result with the known genus was used and noted.

3.5. STATISTICAL ANALYSIS

Linear regressions were used to examine the relationship between ergosterol and microbial activity on leaves from the sites. Results from the sites were compared using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Variables were log-transformed as necessary to meet assumptions of parametric statistics. Statistics were performed with SigmaStat software (version 2.03). Results were considered significant if P was < 0.05.

Nonmetric multidimensional scaling (NMDS) was used with PC-ORD to ordinate the fungal community data (from DGGE phylotypes) into fewer dimensions. The ordination was based on Sorensen's distances among composition data (proportional) after arcsin-squareroot transformation. Our two-dimensional NMDS solution had a stress value of 0.18, which was significant when tested against Monte Carlo simulations. We correlated the 2 NMDS axis scores for the 11 sites with site characteristics and proportion of the most common taxa.

4. RESULTS

4.1. HYDROLOGY OF SITES

AYSDry and AYSWet were continuously immersed in isolated pools in the intermittent section. YBPL was continuously immersed in a run. KTWet, TTWet, BBWet, and QuarWet were continuously immersed in riffles. All sites, including Dry sites, were immersed on days 8, 36, and 81 after precipitation events. KTDry and BBDry were also fully immersed on days 43, 63, and 72. TTDry and QuarDry were wet as above as well as days 13, 30, and 93. All dry sites (with the exception of AYSDry) were emersed again within 48 hours after flooding.

4.2. WATER QUALITY OF SITES

The water quality parameters remained within an expected range (Table 4.1). Temperature and conductivity ranges were similar among sites. Dissolved oxygen was near saturation. SRP (Soluble Reactive Phosphorus) concentrations were low (less than 6 µg/L) at all sites. DIN (Dissolved Inorganic Nitrogen) concentrations were more variable, with one site having DIN of 213 µg/L while others were less than 60 µg/L.

Table 4.1. Water quality of the streams at the study sites.

Site	Temperature (°C)	Conductivity (µS/cm)	DO (mg/L)	SRP (µg/L)	DIN (µg/L)
AYS	6.8 - 9.7	280-324	7.5-14.4	3.7	13
YBPL	7.6 - 13	262-285	7.4-14.5	5.2	213

Table 4.1. Water quality of the streams at the study sites (continued).

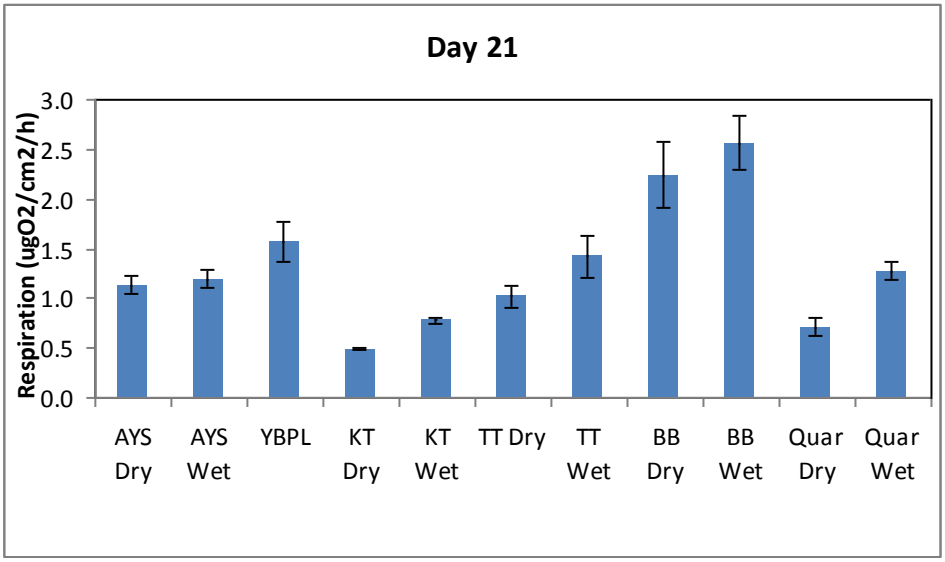
KT	6.7 - 10.4	174-340	6.3-12.75	3.1	54
TT	3.8 - 8.6	260-414	7.8-14	3.4	51
Quar	1.1 - 8	172-266	8.2-15.7	n/a	n/a
BB	2.1 - 8.2	195-335	7.8-19.7	n/a	n/a

4.3. RESPIRATION AND ERGOSTEROL

Respiration rate on leaves varied from less than 0.5 to over 4 μg O_2 cm^{-2} h^{-1} (Figure 4.1). During the first sampling on day 21, dry and pool sites had lower respiration rates than the corresponding wet sites. During later samplings (days 62, 83, and 104), there was no clear pattern between wet and dry sites. Certain sites, such as the BB sites, had higher respiration than other sites throughout the study.

Respiration was closely related to ergosterol across all sampling sites and times (Figure 4.2). Ergosterol accounted for about half of the variation in respiration on the decomposing leaves.

(A)



(B)

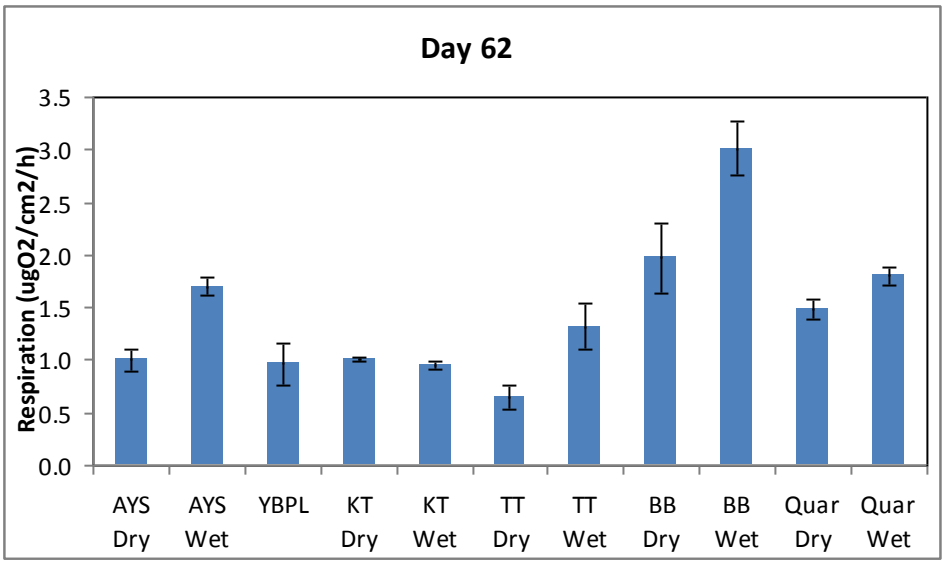
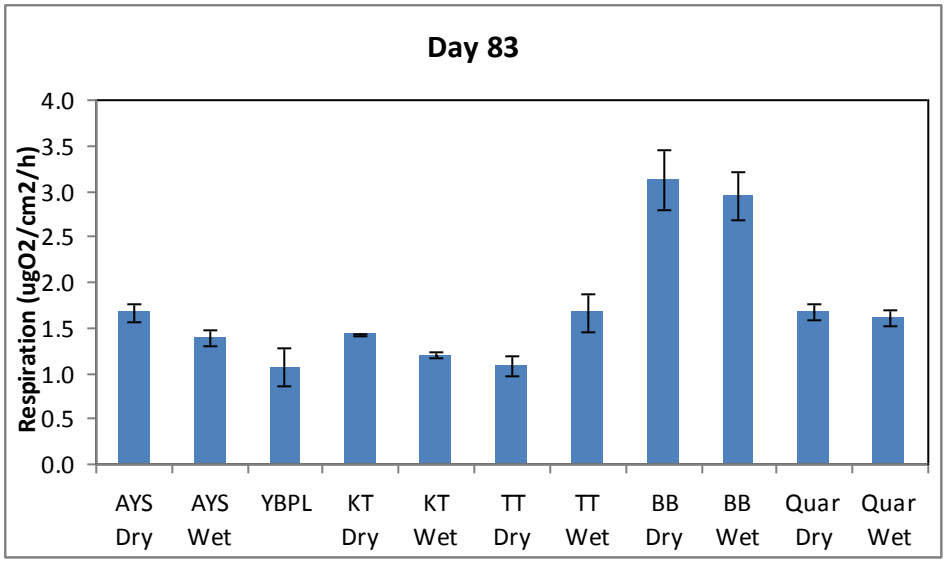


Figure 4.1. Respiration rates on decomposing leaves among sites for the four sampling times (A) through (D).

(C)



(D)

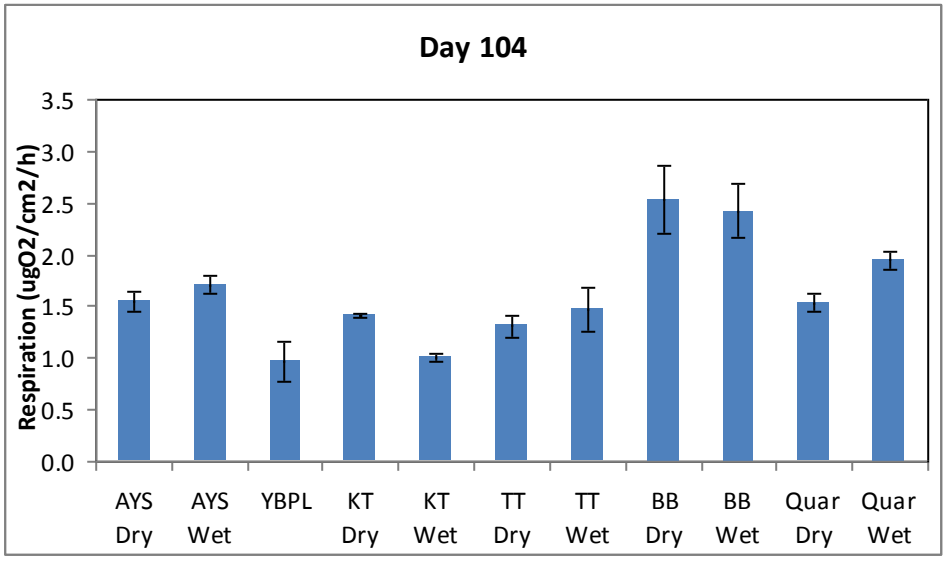


Figure 4.1. Respiration rates on decomposing leaves among sites for the 4 sampling times (A) through (D) (continued).

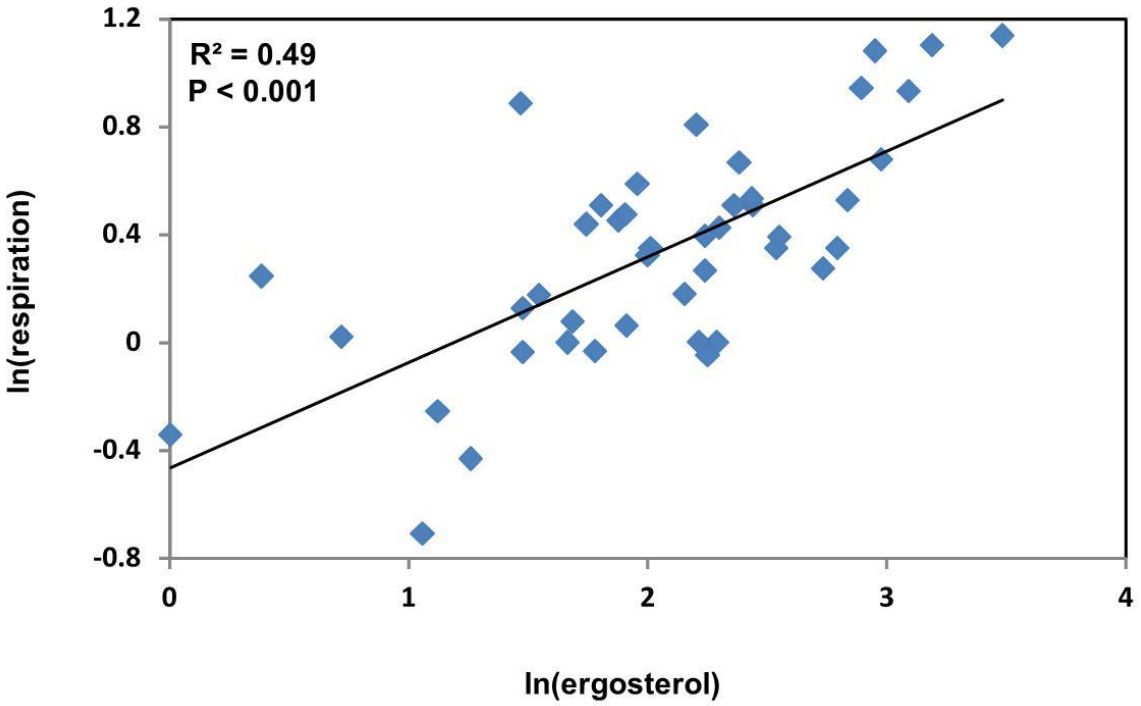


Figure 4.2. Respiration of decomposing leaves versus ergosterol as a measure of fungal biomass. Data are ln-transformed to ensure normally distributed data. Line is regression line with statistical results shown.

4.4. DIVERSITY OF FUNGAL COMMUNITIES

4.4.1. Diversity Assessment. In general, DGGE gels had fewer phylotypes, indicating likely lower species richness, during the first sampling (Day 21) compared to later samplings (see DGGEs in Appendix). Also, the Shannon-Wiener diversity index for DGGE profiles was calculated for the Day 83 sampling (Figure 4.3). Diversity ranged from 1.02 for the TT Dry site to over 2 for both Quar sites. For 3 of the 5 site comparisons, the emersed or pool sites had lower diversity than the immersed sites.

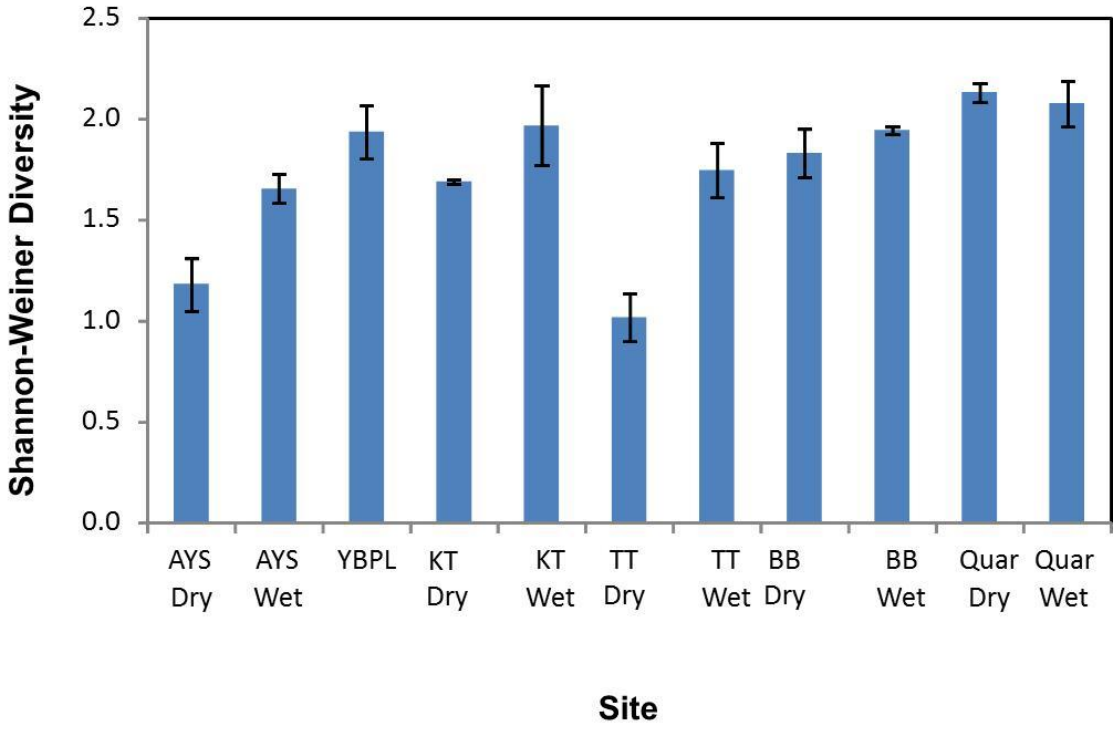


Figure 4.3. Shannon-Weiner diversity of fungal communities from Day 83 sampling.

4.4.2. Ordination of Fungal Communities. The fungal community data from DGGE profiles of the Day 83 sampling was also ordinated using NMDS (Figure 4.4). Triplicate samples tended to group together, although 2 sites (TT Dry and TT Wet) had outliers with varying communities. Wet and dry sub-sites tended to group close to each other. Wet and dry sites were separated along Axis 2 of the ordination.

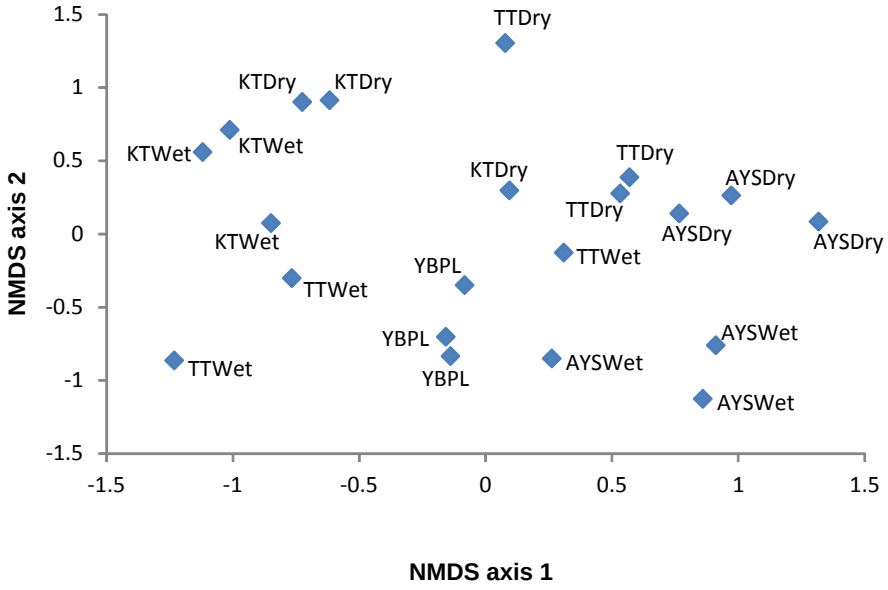


Figure 4.4. Nonmetric multidimensional scaling ordination of fungal communities based on DGGE phylotypes from Day 83 sampling. Points with same site name are triplicate samples. Two-dimensional ordination was based on Sorenson's distance matrix. Stress value was 0.18.

4.5. COMMUNITY STRUCTURE

4.5.1. Community DGGE. All environmental DGGEs had complex banding patterns. For almost all sites Day 21 had a simpler pattern than the following time points. All DGGEs had bands that appeared, for the first time, after Day 21. The vast majority of bands present were very faint compared to a smaller number of dominant bands. Their presence was not incidental as these fainter bands are often seen spanning many replicates and time points. Several species appeared at multiple migration distances; for example in Figure 4.5 (A) *Alternaria* sp. is seen in bands 2 and 3. These two *Alternaria* species have different accession numbers. In other environmental gels (see appendix) *Penicillium* sp. is seen appearing at different migration distances, even though the

accession numbers for each are the same. No bands of the same migration distance are caused by more than a single species in our data set. *Penicillium* sp. is by far the most common and dominant species in our trial. In lane "c" of Day 21 in the YBPL gel, where it appears as one of its weakest lanes, it still accounts for ~ 12.5% of the community. All species of *Penicillium* sp. matched the same accession number. In Figure 4.6 individual clones are compared using DGGE with their library's original environmental sample to identify corresponding bands.

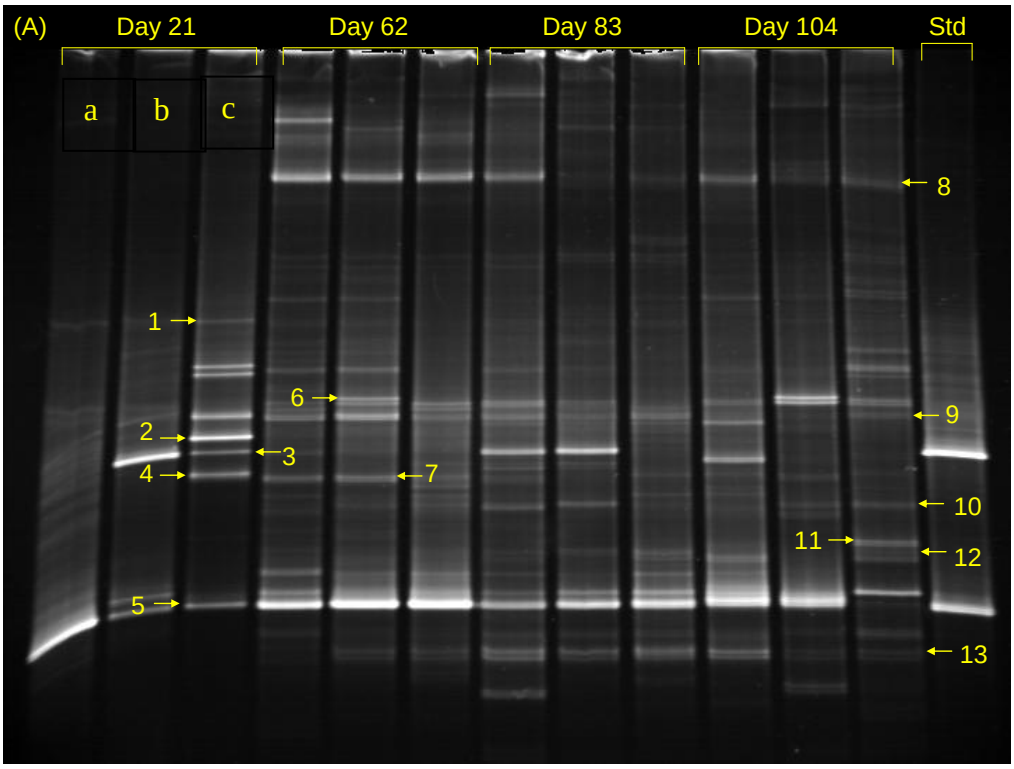


Figure 4.5. YBPL Environmental DGGE (A). For each time point triplicates samples a, b, and c are left to right. **Std** is a relatively simple sample used in all environmental gels to act as a ladder. (**1**)*Pythium sp.* (**2**)*Alternaria alternata* (**3**)*Alternaria sp* (**4**)*Epicoccum sp.* (**5**)*Penicillium sp.* (**6**)*Leptodiscella sp.* (**7**)*Pythium sp.* (**8**) *Pythiogeton sp.* (**9**)*Pestelotiopsis sp.* (**10**)*Lunulospora curvula* (**11**) *Helotiales sp. (possibly Articulospora tetracladia)* (**12**)*Alatospora acuminata* (**13**)*Stachybotrys chartarum.*

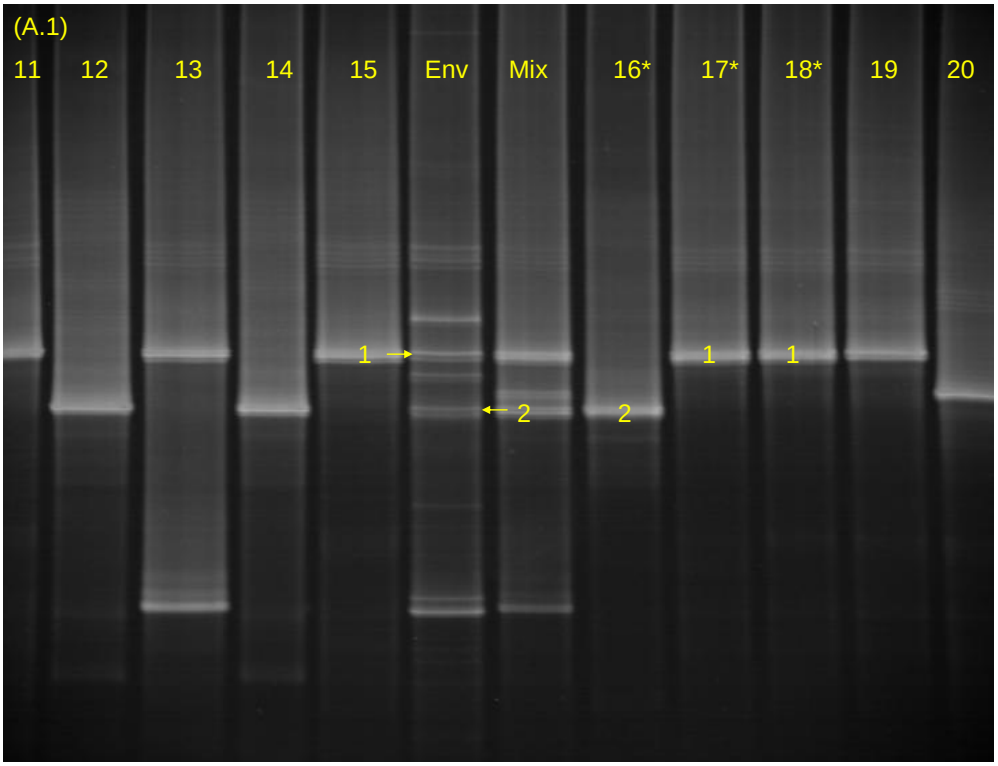


Figure 4.6. YBPL 12-4-11 C Clones 11-20 (A.1). A single clone library DGGE associated with the above gel. **Env** is the respective environmental DNA extraction (in this case the second lane [b] of the Day 62 time point.) **Mix** is a composition of 1µl of each of the clones (11-20). Lane numbers with an asterisk were sequenced. (**1**) *Alternaria alternata* (**2**) *Epicoccum sp.* (These correlate to bands 2 and 4 respectively in the above environmental DGGE). Additional YBPL clones are in the Appendix.

4.5.2. Clone Library Analysis. Approximately 200 clone colonies were selected and run on DGGE for comparison to environmental samples. Of these 50 were selected for sequencing, as seen in Table 4.2. Not all major bands, and only a small portion of minor bands resulted in clone bands that matched and had little additional banding. In almost every clone library DGGE, there was a band in the mixed sample that did not match any apparent environmental sample. These bands should not be discounted because they often match bands in neighboring lanes, even months later. In Table 4.2 underlined species are aquatic hyphomycetes. *Massarina and Articulospora* were the first identified hit from the BLAST search. In the case of any other Order or higher

identification, no Genus or closer level hit was available. It is worth noting that all *Penicillium* sp. share the same accession number. (Asterisks indicate a listing with a second, defined, hit in Gen Bank.)

Table 4.2. BLAST results.

Clone Name	*Tentative genus from BLAST*	Max Identity	Accession number
TTDry 1-14-12A Clone 8	*Penicillium sp.*	99%	KC797645.1
AYSWet 12-4-11B Clone 1	*Penicillium sp.*	99%	KC797645.1
AYSWet 12-4-11B Clone 4	*Penicillium sp.*	99%	KC797645.1
AYSWet 1-14-12C Clone 3	*Penicillium sp.*	99%	KC797645.1
AYSWet 1-14-12C Clone 4	*Penicillium sp.*	100%	KC797645.1
YBPL 1-14-12B Clone 4	*Penicillium sp.*	100%	KC797645.1
YBPL 1-14-12B Clone 5	*Penicillium sp.*	100%	KC797645.1
YBPL 1-14-12B Clone 17	*Penicillium sp.*	99%	KC797645.1
TTWet 1-14-12B Clone 6	*Penicillium sp.*	100%	KC797645.1
TTWet 1-14-12B Clone 7	*Penicillium sp.*	100%	KC797645.1
TTWet 1-14-12B Clone 9	*Penicillium sp.*	99%	KC797645.1
TTDry 1-14-12A Clone 7	*Penicillium sp.*	100%	KC797645.1
AYSWet 2-25-12C Clone 6	*Penicillium sp.*	99%	KC797645.1
AYSWet 2-25-12C Clone 7	*Penicillium sp.*	99%	KC797645.1
AYSWet 2-25-12C Clone 8	*Penicillium sp.*	99%	KC797645.1
AYSWet 2-25-12C Clone 9	*Penicillium sp.*	99%	KC797645.1
TTDry 2-4-12A Clone 5	*Pestelotiopsis sp.*	99%	JX624316.1
AYSWet 12-4-11B Clone 2	*Pestelotiopsis sp.*	99%	JX684005.1
AYSWet 1-14-12C Clone 2	*Pestelotiopsis sp.*	100%	JN198506.1
AYSDry 2-25-12C Clone 18	*Pestelotiopsis sp.*	99%	HQ022328.1
YPBL 2-25-12C Clone 13	*Pestelotiopsis sp.*	99%	JX999998.1
AYSWet 2-25-12C Clone 5	*Pestelotiopsis sp.*	100%	JX684005.1
AYSWet 12-4-11B Clone 3	*Alternaria sp.*	99%	JX960579.1
AYSWet 12-4-11B Clone 5	*Alternaria sp.*	100%	KC460834.1
AYSWet 12-4-11B Clone 6	*Alternaria sp.*	100%	KC771501.1
AYSDry 1-14-12B Clone 13	*Alternaria sp.*	100%	KC771501.1
YBPL 12-4-11C Clone 8	*Alternaria sp.*	100%	KC771501.1
YBPL 12-4-11C Clone 10	*Alternaria alternata*	100%	FJ441003.1
YBPL 12-4-11C Clone 17	*Alternaria sp.*	99%	JQ306333.1
YBPL 12-4-11C Clone 18	*Alternaria sp.*	100%	JQ306333.1
YBPL 1-14-12B Clone 16	*Alternaria sp.*	99%	KC460834.1
AYSDry 1-14-12B Clone 16	*Alternaria aborescens*	100%	KC464334.1
AYSWet 2-25-12C Clone 4	*Phoma sp.*	99%	KC357255.1
AYSDry 1-14-12B Clone 15	*Phoma sp.*	100%	AB746934.1
AYSWet 2-25-12C Clone 10	*Phoma fungicola*	100%	KC357255.1
AYSDry 1-14-12B Clone 14	*Epicoccum sp.*	99%	HE681443.1

Table 4.2. BLAST results (continued).

YBPL 12-4-11C Clone 16	*Epicoccum sp.*	99%	KC106717.1
YBPL 1-14-12B Clone 3	*Leptodiscella sp.*	99%	FR821312.1
YBPL 1-14-12B Clone 6	*Stachybotrys chartarum*	99%	KC305355.1
YPBL 2-25-12C Clone 7	*Alatospora acuminata*	96%	AY204590.1
YPBL 2-25-12C Clone 11	*Alatospora acuminata*	98%	AY204590.1
YPBL 2-25-12C Clone 18	*Lunulospora curvula*	98%	AY729938.1
TTDry 2-4-12A Clone 7	*Tetracladium sp.*	100%	GQ411300.1
TTDry 2-4-12A Clone 8	*Tetracladium sp.*	100%	GQ411300.1
YPBL 2-25-12C Clone 8	*Pythiogeton sp.*	93%	JQ610187.1
AYSDry 2-25-12C Clone 19	*Pythium sp.*	82%	JF431916.1
YBPL 12-4-11C Clone 2	*Pythium sp.*	95%	HQ643816.1
YBPL 1-14-12B Clone 8	*Pythium sp.*	96%	HQ643816.1
YBPL 1-14-12B Clone 9	*Phythium boreale*	100%	HQ643372.1
TTDry 1-14-12A Clone 2	*Undescribed Fungal Sp.*	91%	HQ392612.1
TTDry 1-14-12A Clone 3	*Undescribed Fungal Sp.*	91%	HQ392612.1
YPBL 2-25-12C Clone 4	*Helotiales sp.*	99%	JX001616.1
YPBL 2-25-12C Clone 6	*Ascomycota sp.*	90%	JN850997.1
*AYSDry 2-25-12C Clone 14	*Ascomycota sp.*	89%	FJ375157.1
**TTWet 1-14-12B Clone 1	*Uncultured Ascomycota*	99%	HM141057.1
*AYSDry 2-25-12C Clone 14	*Massarina sp.*	88%	*DQ863675.1
**TTWet 1-14-12B Clone 1	*Articulospora tetracladia*	95%	**EU998924.1

5. DISCUSSION

5.1. IMPLICATIONS

Aquatic fungi appear to be sensitive to many anthropogenic stressors, including acidity, metals, nutrients, and organic contaminants (Gessner and Chauvet 2002, Krauss et al. 2011). The effects of "dry" versus "wet" conditions at my study sites revealed only minor differences among these treatments. One interesting conclusion from this research is that stream fungi may be relatively tolerant to emersion, at least for relatively short time periods.

There were some differences among sites based on hydrology at the first sampling time. At each site within a dry-wet pair, the microbial activity was higher at the wet site at Day 21. This initial difference likely reflects different rates of colonization between the immersed sites in flowing water and the emersed or pool sites. Leaves in the stream would be subjected to a constant source of spores and hyphal fragments that could quickly colonize the sterile leaves. The dry sites could be colonized from the soil, the air, or from the intermittent immersion during high flows.

Over time the differences became less pronounced and might even show a slight trend towards higher activity at the dry sites. Maamri et al. (2001) noticed a similar "catching up" period of higher than normal activity when water was reintroduced to a previously dry environment. This implies that in environments with wetting-drying cycles, the leaves are likely to still completely break down, but more of that process will be shifted away from the major abscission events in fall and drawn out towards spring.

This could impact the life cycle stages of shredding invertebrates that have more time sensitive life cycles. It also implies that leaves which become emersed after they are well under way in the breakdown process are not likely to be heavily impacted.

This difference in activity is likely not due to the presence or lack of any specific fungal species but rather due to overall fungal biomass. Ergosterol measurements generally support this. This finding supports the idea of functional redundancy, where the specific taxa present are not as important as the overall biomass in determining activity. Experimental studies on fungal diversity and function have supported this as well (Dang et al. 2005).

Although being a dry site or a wet site does affect community structure, it appears that being at a specific site has more impact overall in determining the community composition, at least in regards to this study. The fungal community based on DGGE phylotypes tended to ordinate together by site more so than by wet or dry. It should be noted that the dry sites always remained damp during the study, given the intermittent precipitation and cool weather during winter. Harsher drying conditions, or sites further from the stream channel, could have more dramatic effects on the community, and eventually may begin to outweigh location for determining community structure. In light of the clone library findings, this seems less likely, however. Many of the species that appear to already be dominant in immersed sites are in fact considered to be “terrestrial” fungi rather than “aquatic”.

The clone library did identify several major bands, and a few minor bands within the environmental DGGEs. None of the major bands turned out to be aquatic hyphomycetes. Terrestrial fungi have been found repeatedly in streams (Bärlocher and

Kendrick 1974); our results show that they might not just be present but actually dominate the community. Several genera (*Alternaria, Penicillium* and *Pestelotiopsis*) appear early, often, and at high intensity throughout many of the sites. This comes as a surprise as these bright common bands would have classically been assumed to be good candidates for aquatic hyphomycetes. The bands that did match aquatic hyphomycetes were common in many sites and time points, but were always minor bands when comparing intensity. It can be difficult to interpret banding patterns across multiple DGGEs, but one interesting point is that the aquatic hyphomycete bands do not appear to be significantly more intense in the wet sites than their dry counterparts.

The dominance of *Penicillium* sp. is very surprising. The very fact that it is missing in some samples while remaining so constant throughout the rest suggests a confounding factor for that particular leaf core rather than an inability for the *Penicillium* sp. to simply migrate there.

As previously mentioned, three other papers that have used clone libraries to assess aquatic fungi have found that the majority of species were either undetermined or not aquatic hyphomycetes (Seena et al. 2008, Harrop et al. 2009, Kelly et al. 2010). These studies support the findings of my research.

5.2. LIMITATIONS

Determining the presence of a fungus, even commonly found on decaying leaves does not prove its role or value in the ecosystem. Conidial production can be quite high; the energy requirements to produce these numbers of spores can require a significant percentage of the leaf mass lost during the period (Suberkropp 1997). This appears to be

the central evidence that the entire concept of aquatic hyphomycetes dominating leaf litter decomposition in streams is based on. Findlay and Arsuffi (1989) found a maximum of 4% of leaf mass loss converted to conidia over thirty days. However, Sridhar and Bärlocher (2000) found up to ~19% of leaf mass loss was attributable to conidial mass. The streams that these findings were in could be quite different environments that those found in the Ozarks region. It is possible that while aquatic hyphomycetes do tend to dominate in other streams, they are not as important here.

Our study period was much longer than many other community assessment studies. The implications of this approach for our study compared to others are not clear. It could mean that early colonizers are aquatic hyphomycetes, which begin the breakdown process within a few days of the leaf entering the water only to be almost completely displaced by day 21. Nikolcheva et al. (2005) found that leaves had very high fungal diversity after 2-3 days of immersion, most likely due to arboreal fungi carried into the stream with the leaf as it fell in addition to colonizing stream fungi. These arboreal species were then lost after several weeks as they were replaced by aquatic hyphomycetes. Our results imply that those species are either replaced themselves by other species, or may not play as large a role as previously assumed in the later stages of leaf decay. The first possibility seems less likely as many of the aquatic hyphomycetes in the clone library tended to match bands that became increasingly intense over time.

There are two major shortcomings of using these molecular approaches to community assessment. First, rDNA is the target of our primers, and it can remain present in a sample long after the fungi have become metabolically inactive if it is adsorbed to clay (Blum et al. 1997). This might mean that there is some ghost banding

from previously active fungi in some of the later DGGE analyses. This bias might explain some of the increase in apparent diversity in later samples. However, many of these bands appeared over the course of several months, with little intensity loss, suggesting that the DNA was not slowly degrading but was in fact being extracted from active fungi.

The second shortcoming involves bias in amplification of mixed DNA from community sampling. PCR bias has been shown to play only a small if not insignificant role in environmental samples, where the diversity of the sample is enough that no single template reaches saturation concentration (Suzuki et al. 1998). However, gene copy number could still be an issue. If the ribosomal gene has an exceptionally large copy number in certain species, they might appear to be more dominant than they actually are. However, Nikikcheva et al. (2003) found that when mycelia of two species were mixed in known ratios, band intensity in DGGE approximated the ratios of the mixtures. In addition, rDNA copy number for fungi generally range from 50 to 220, which means that the magnitude of a band from equal cell counts could have as high as a 4 fold difference between the species.

One final critique of DGGE is the point that a single band could be multiple species or that multiple bands could represent a single species. We did find evidence of the latter. However, the most common band throughout the study was always *Penicillium* sp. and no other species matched that band. This implies that single bands can represent single species.

5.3. FUTURE RESEARCH

Overall, there was enough of an impact of the drying cycles to make a significant impact on community structure and function, but in neither case was the impact profound when compared to other stressors previously studied. The “dry sites” in this study were always damp. Stream fungi may have an innate tolerance to drying, or the damp conditions may have enabled survival of the fungi. Exposure to more complete drying may lead to different effects on fungal communities and activity.

It is also unclear why a completely submerged leaf would have much higher microbial activity than a moist counterpart at a mechanistic level. A clearer understanding of this activity could help define what “wet” environments and “dry” environments truly are from a fungal prospective.

APPENDIX

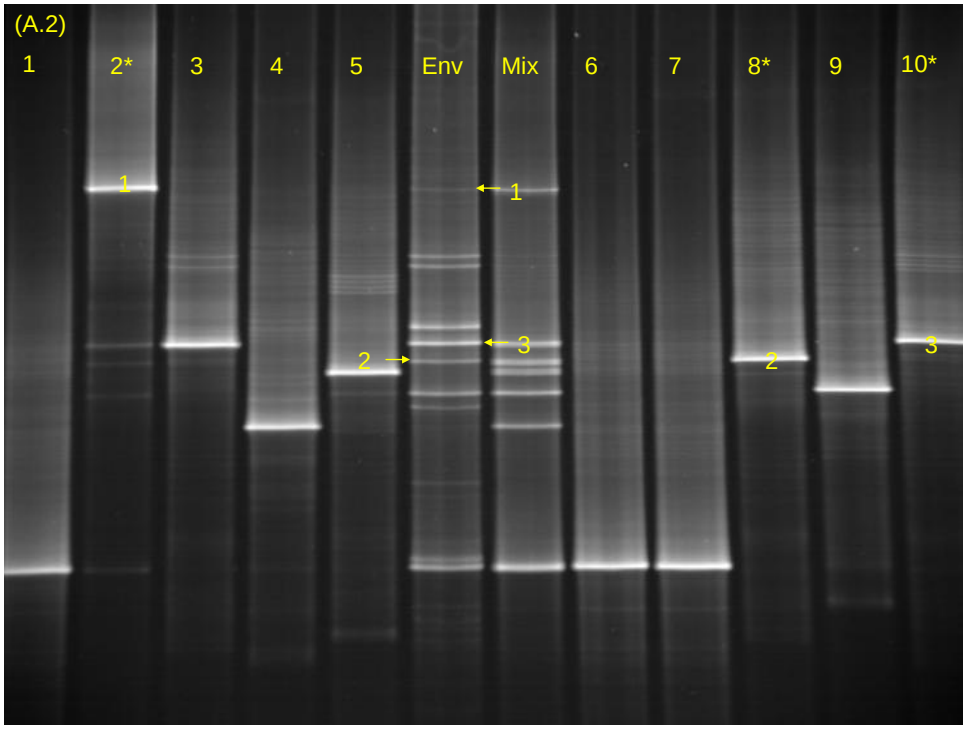


Figure A-1. YBPL 12-4-11 C Clones 1-10 (A.2). Fig 4.5.1 continued. (**1**) *Pythium sp.*; (**2**) *Alternaria sp.*; (**3**) *Alternaria alternata*

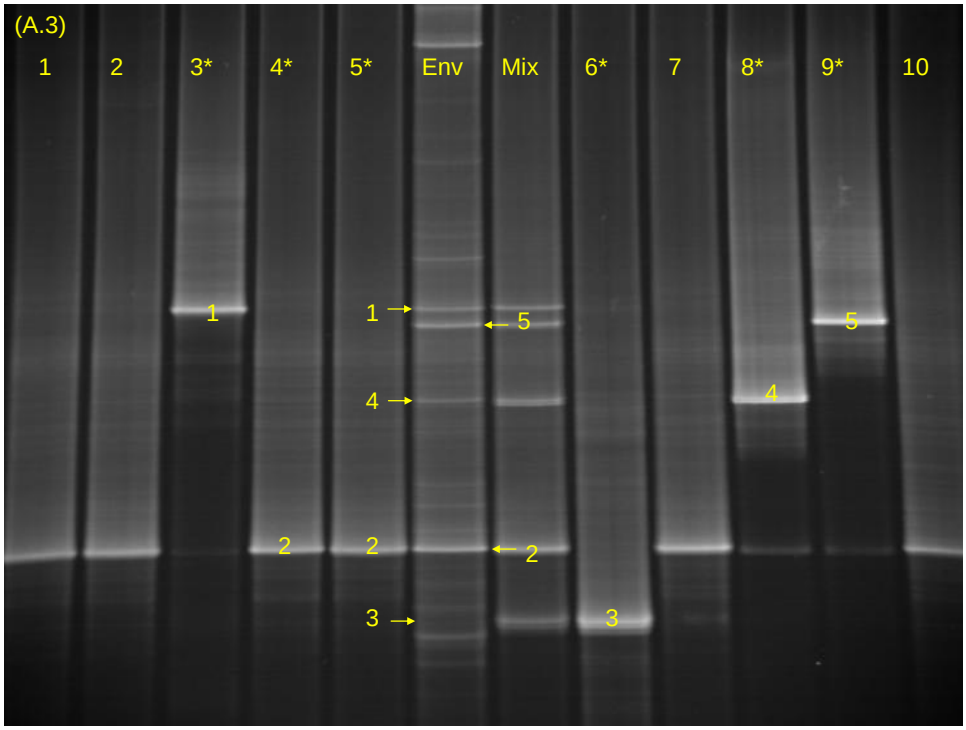


Figure A-2. YBPL 1-14-12 B Clones 1-10 (A.3). (**1**) *Leptodiscella sp.*; (**2**) *Penicillium sp.*; (**3**) *Stachybotrys chartarum*; (**4**) *Pythium sp.*; (**5**) *Pythium boreale*

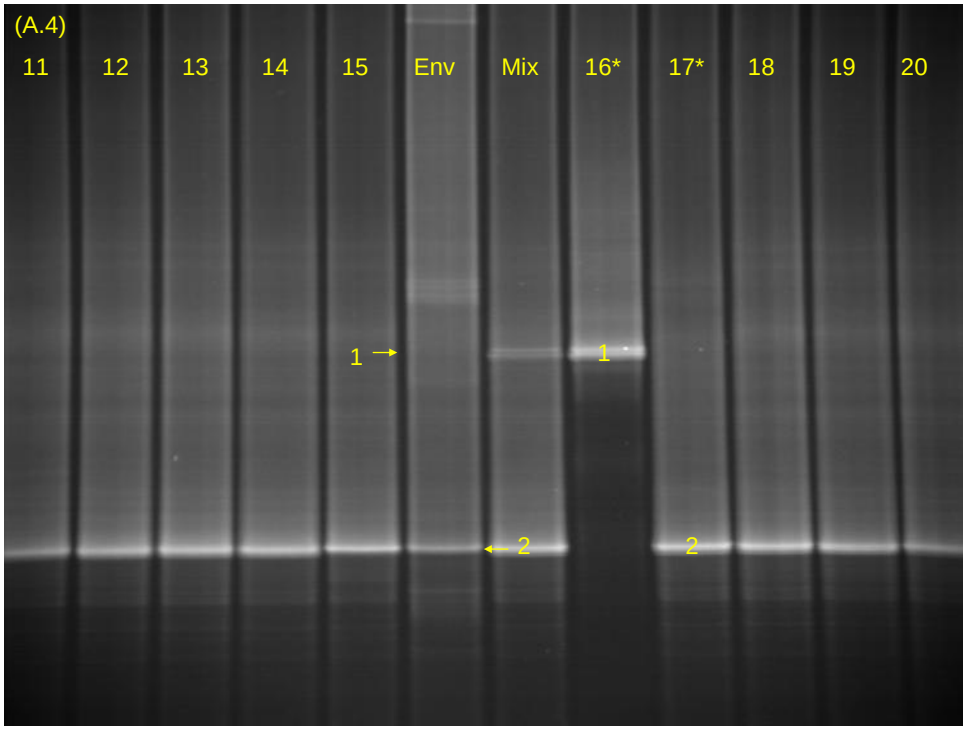


Figure A-3. YBPL 1-14-12 B Clones 11-20 (A.4). (**1**) *Alternaria sp.*; (**2**) *Penicillium sp.*

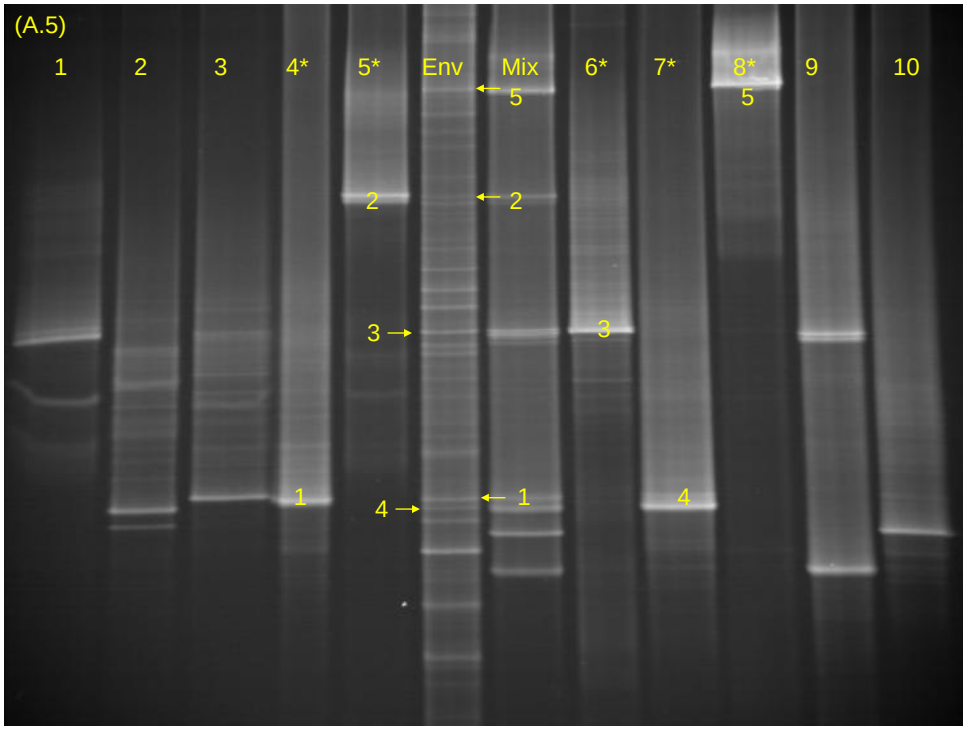


Figure A-4. YBPL 2-25-12 C Clones 1-10 (A.5). (**1**) *Helotiales sp*.; (**2**) Failed sequence. (**3**) *Ascomycota sp*.; (**4**) *Alatospora Acuminata* (**5**) *Pythiogeton sp*.

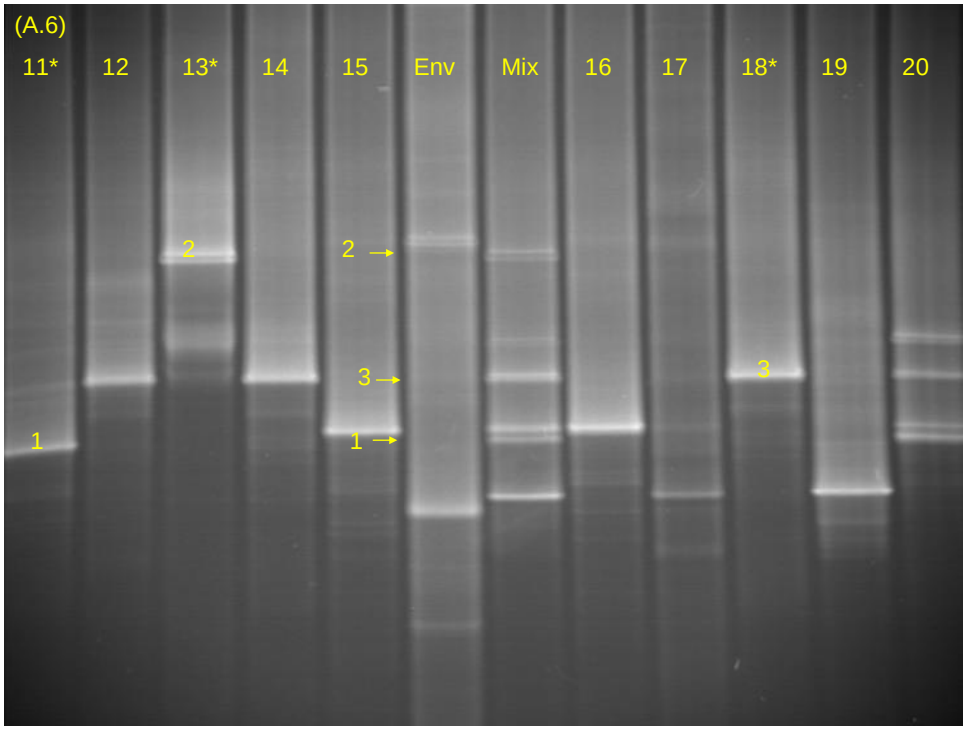


Figure A-5. YBPL 2-25-12 C Clones 11-20 (A.6). (**1**) *Alatospora Acuminata*; (**2**) *Pestelotiopsis sp.*; (**3**) *Lunulospora curvula*

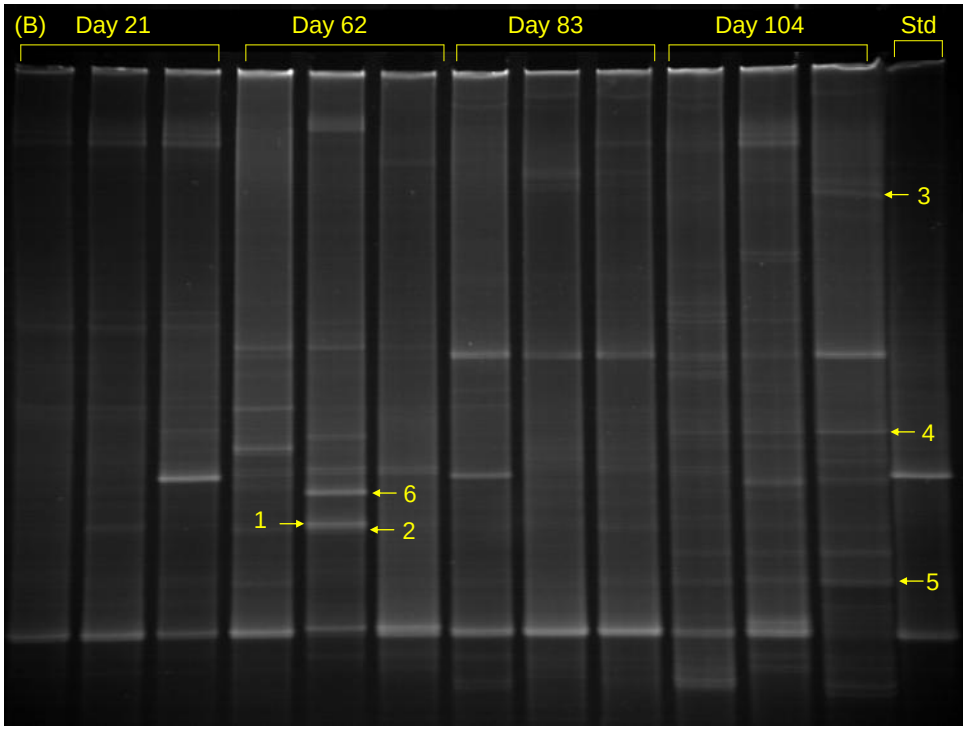


Figure A-6. AYSDry Environmental DGGE (B). (1) *Epicoccum sp.*; (2) *Phoma sp.*; (3) *Pythium sp.*; (4) *Pestelotiopsis sp.*; (5) *Ascomycota sp. (Massarina sp.);* (6) *Alternaria aborescens*

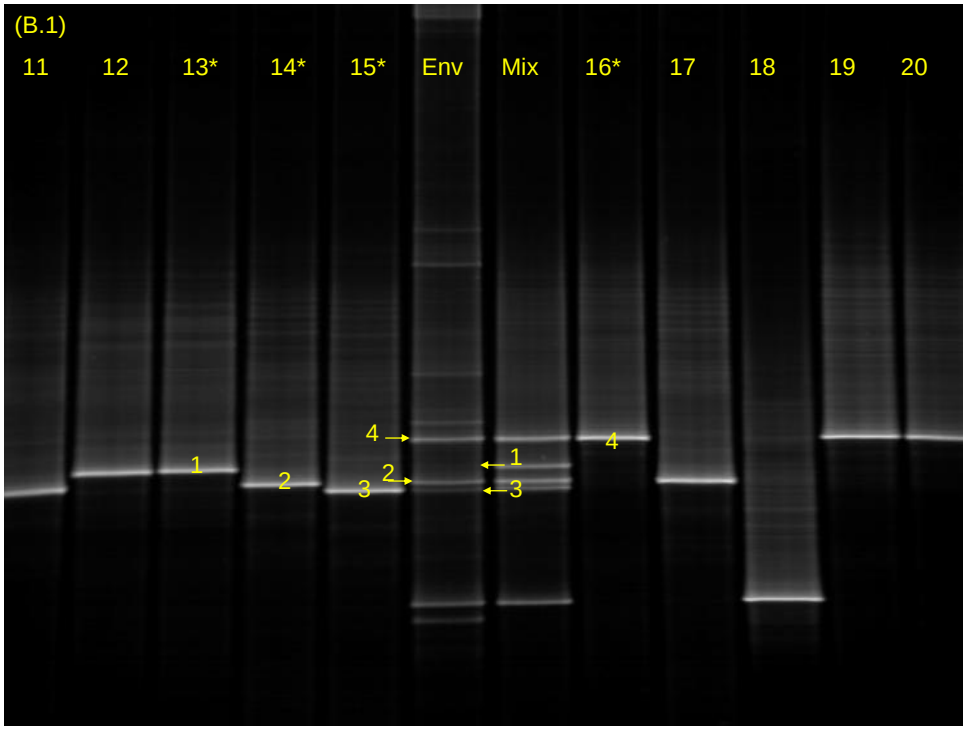


Figure A-7. AYSDry 1-14-12 B Clones 11-20 (B.1). (**1**) *Alternaria sp.*; (**2**) *Epicoccum sp.*; (**3**) *Phoma sp.*; (**4**) *Alternaria aborescens.*

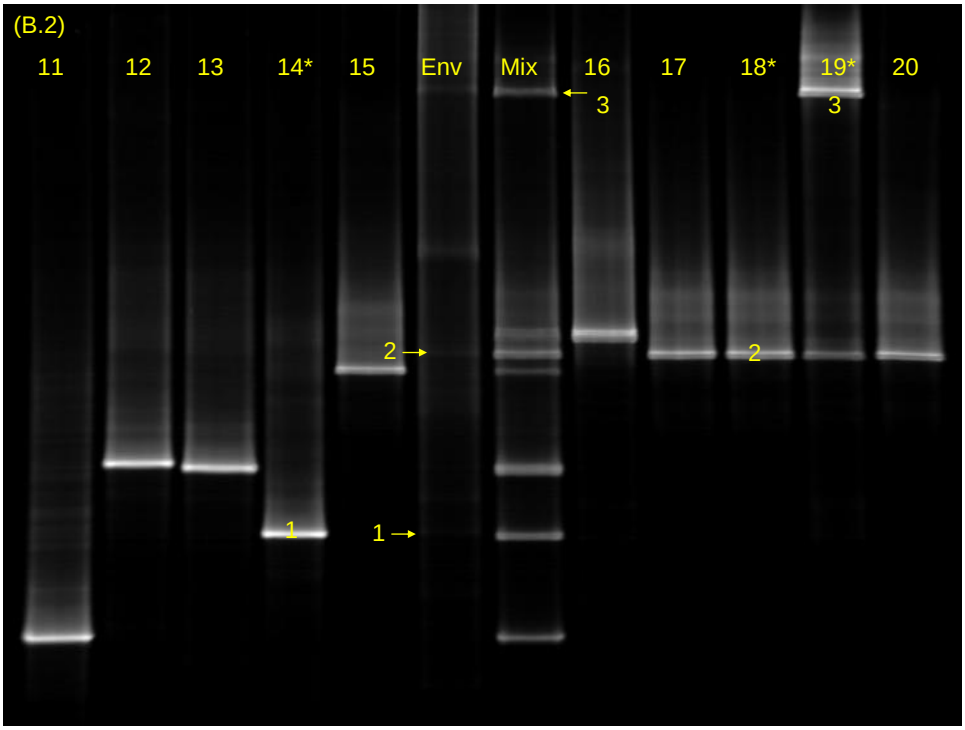


Figure A-8. AYSDry 2-25-12 C Clones 11-20 (B.2). (**1**) *Ascomycota sp. (Massarina sp.);* (**2**) *Pestelotiopsis sp.;* (**3**) *Pythium sp.*

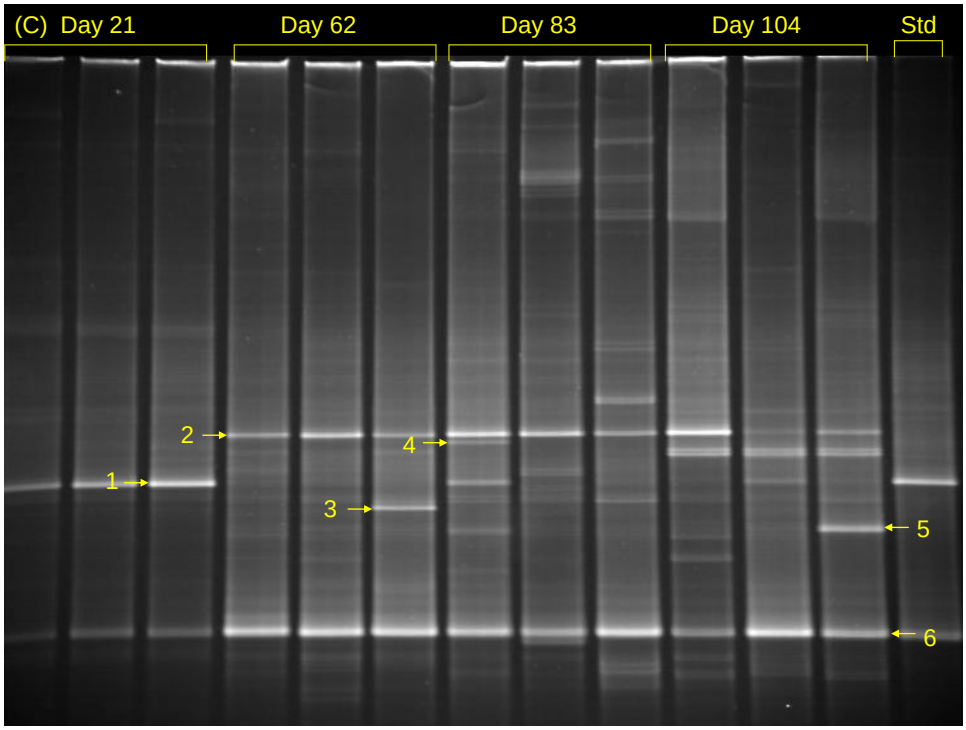


Figure A-9. AYSWet Environmental DGGE (C). (**1**) *Alternaria sp*.; (**2**) *Pestelotiopsis sp*.; (**3**) *Pestelotiopsis sp*.; (**4**) *Alternaria sp*.; (**5**) *Phoma fungicola;* (**6**) *Penicillium sp.*

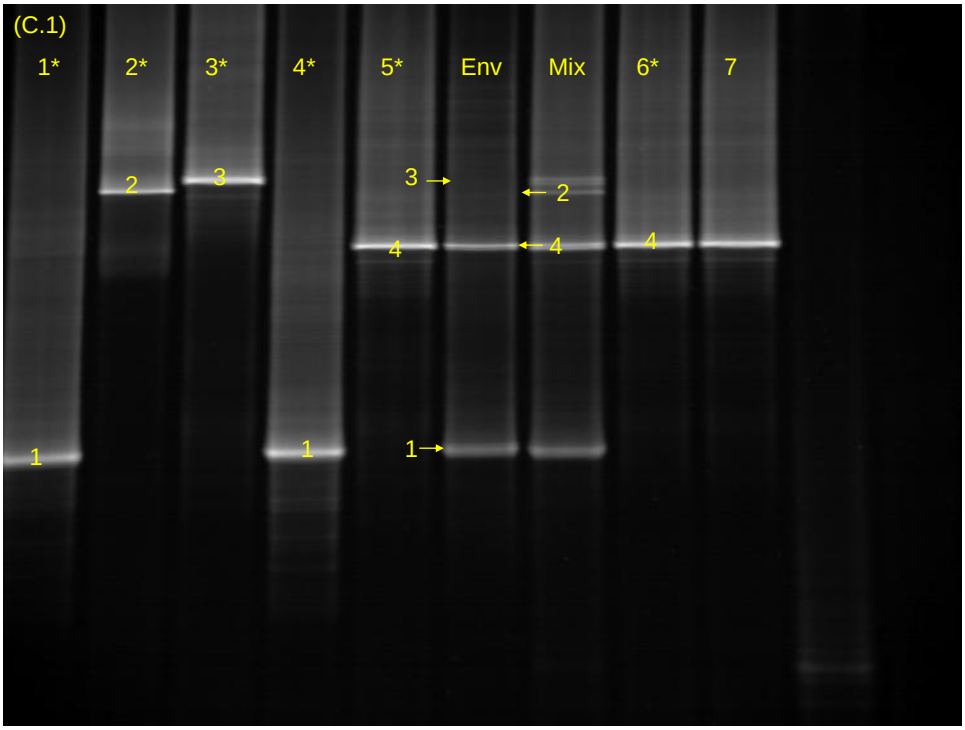


Figure A-10. AYSWet 12-4-11 B Clones 1-8 (C.1). (**1**) *Penicillium sp*. (**2**) *Pestelotiopsis sp*. (**3**) *Alternaria sp*. (**4**) *Alternaria sp.*

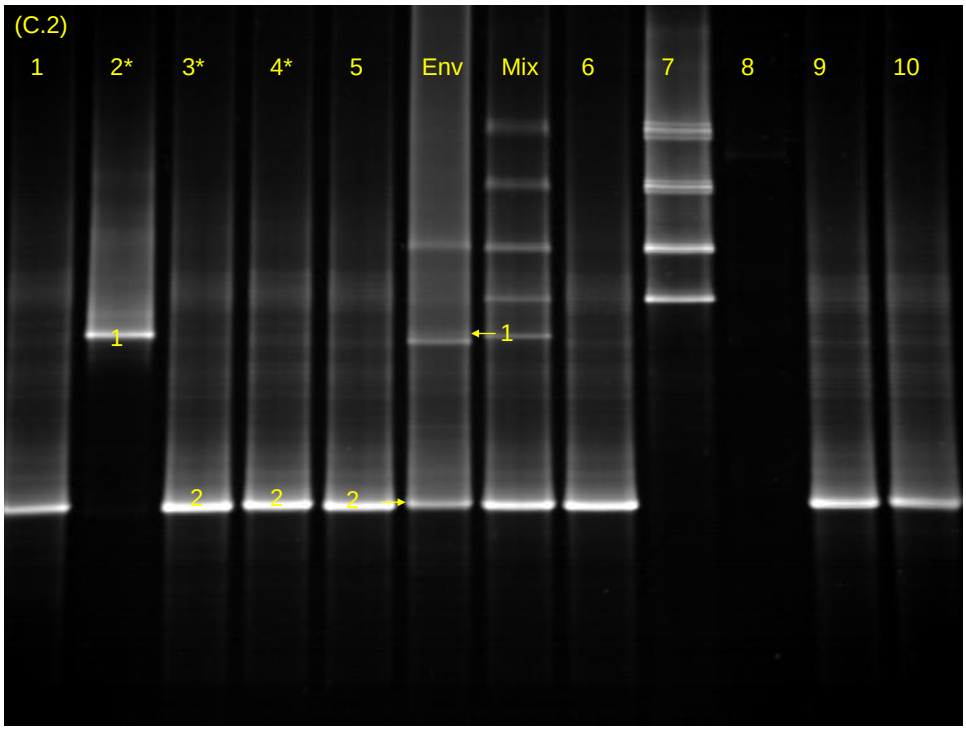


Figure A-11. AYSWet 1-14-12 C Clones 1-10 (C.2). (**1**) *Pestelotiopsis sp*. (**2**) *Penicillium sp.*

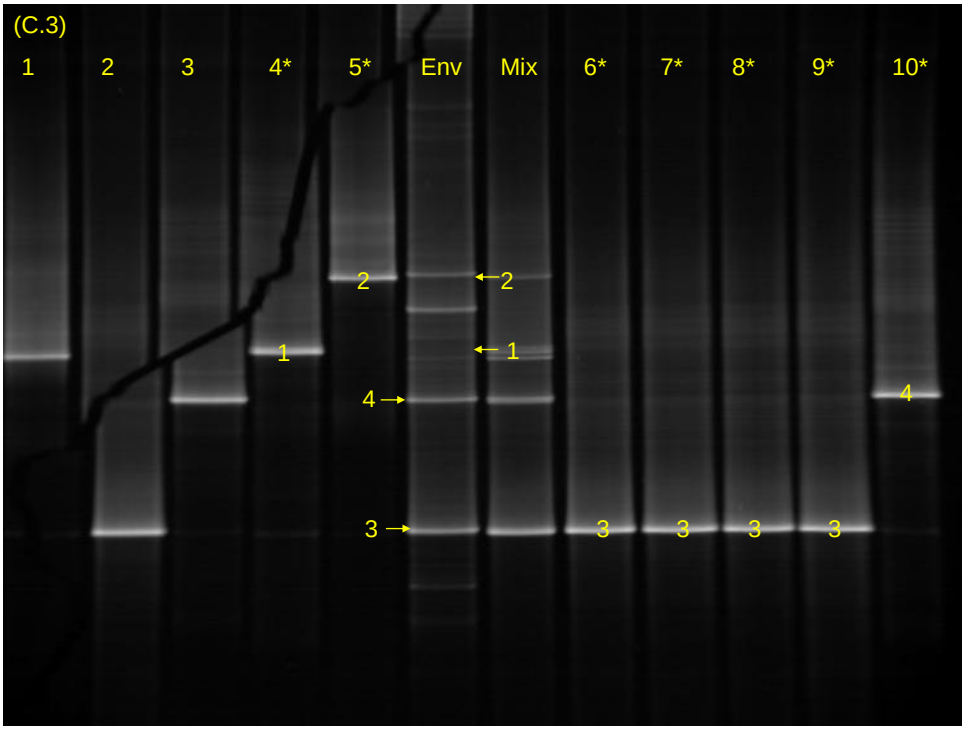


Figure A-12. AYSWet 2-25-12 C Clones 1-10 (C.3). (**1**) *Phoma sp.* (**2**) *Pestelotiopsis sp.* (**3**) *Penicillium sp*. (**4**) *Phoma fungicola*

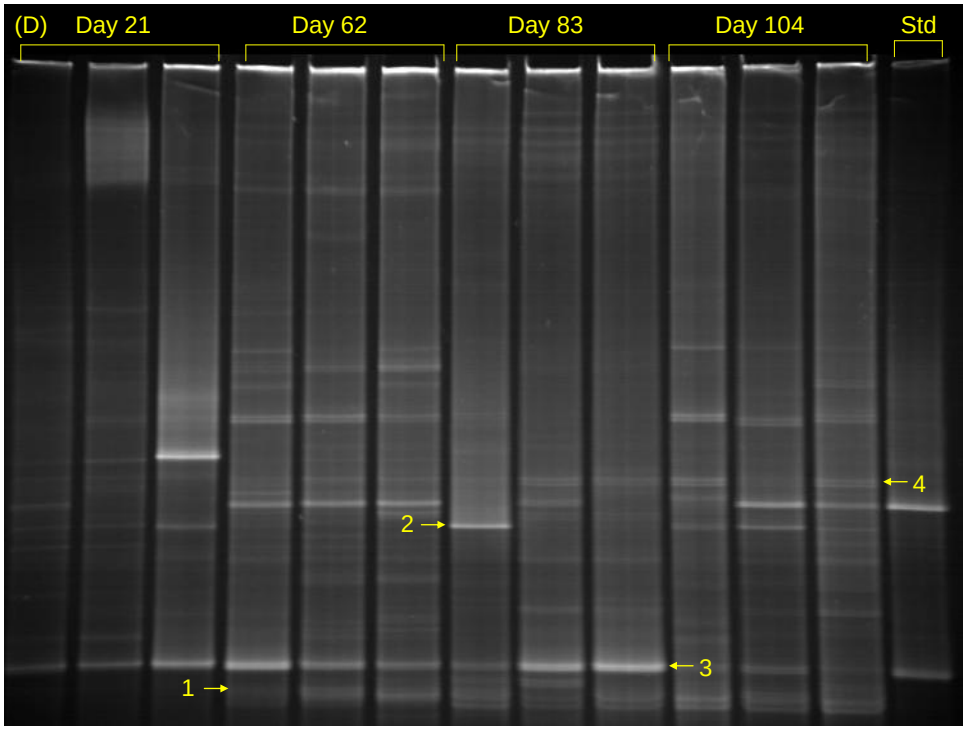


Figure A-13. TTDry Environmental DGGE (D). (**1**) Undescribed Fungal Sp. (**2**) *Pestelotiopsis sp.* (**3**) *Penicillium sp.* (**4**) *Tetracladium sp.*

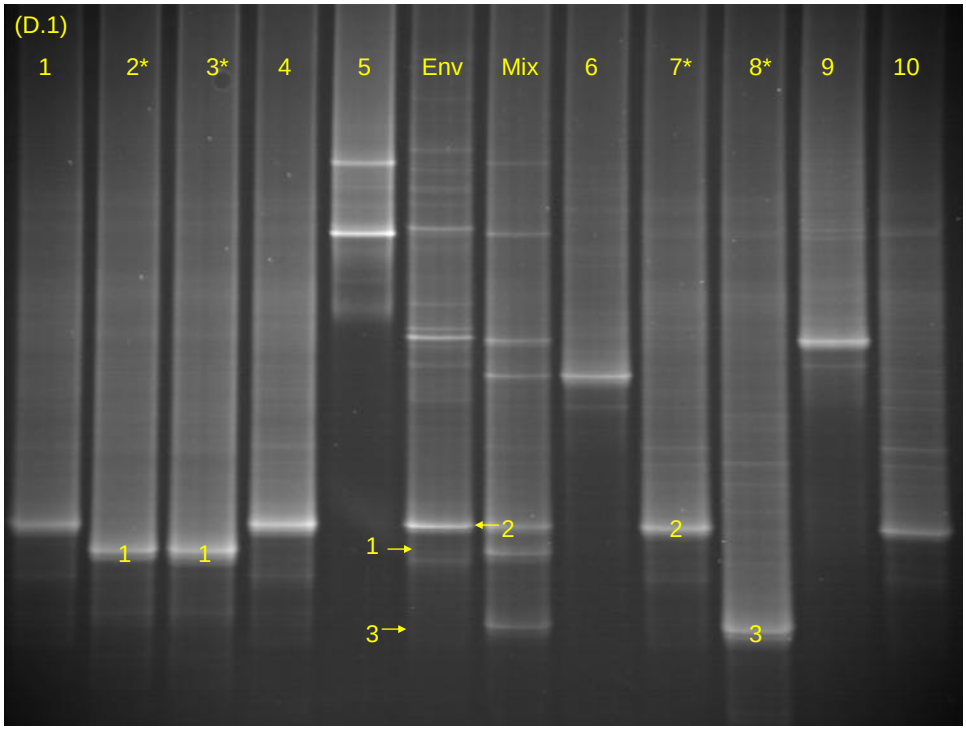


Figure A-14. TTDry 1-14-12 A Clones 1-10 (D.1). (**1**) Undescribed Fungal Sp. (**2**) *Penicillium sp*. (**3**) *Penicillium sp*.

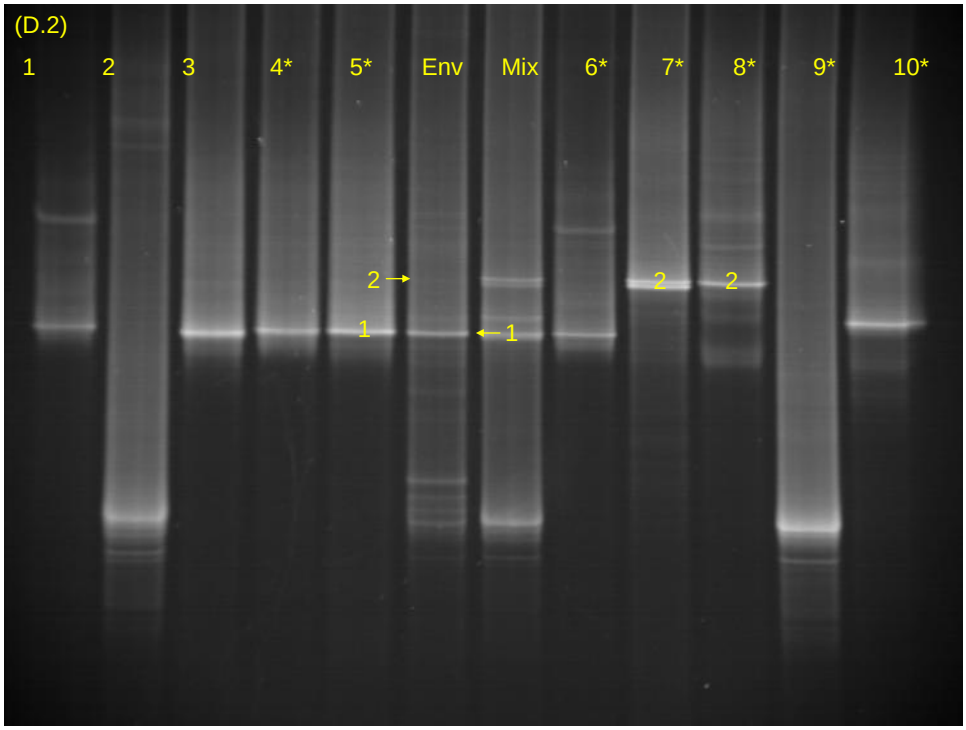


Figure A-15. TTDry 2-4-12 A Clones 1-10 (D.2). (**1**) *Pestelotiopsis sp.* (**2**) *Tetracladium sp.*

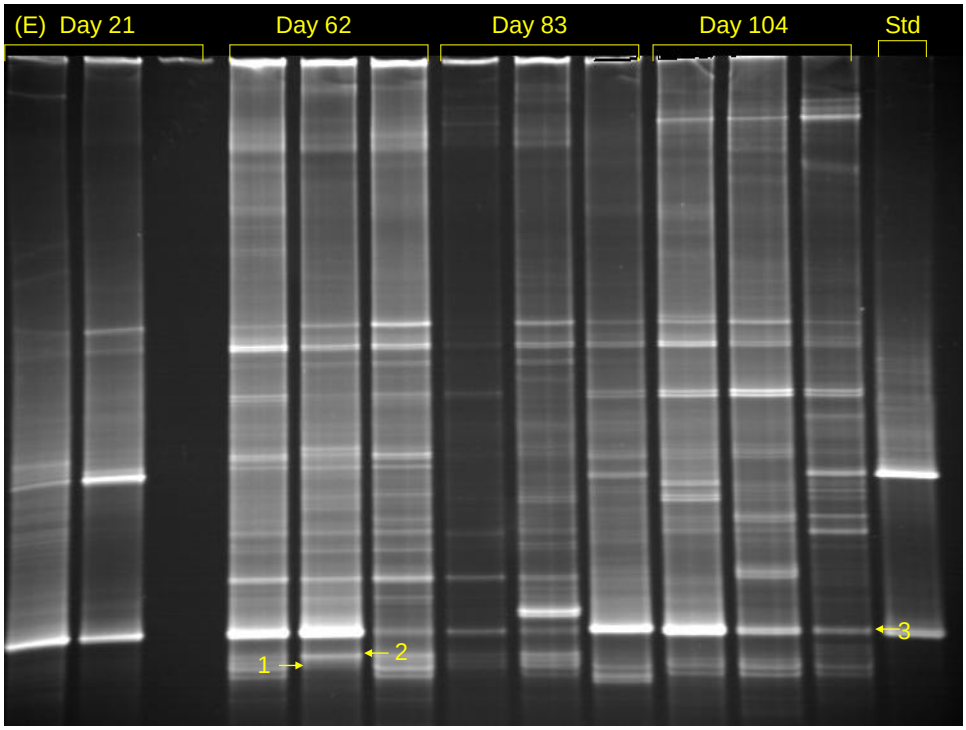


Figure A-16. TTWet Environmental DGGE (E). (**1**) *Penicillium sp.* (**2**) *Articulospora tetracladia* (**3**) *Penicillium sp.*

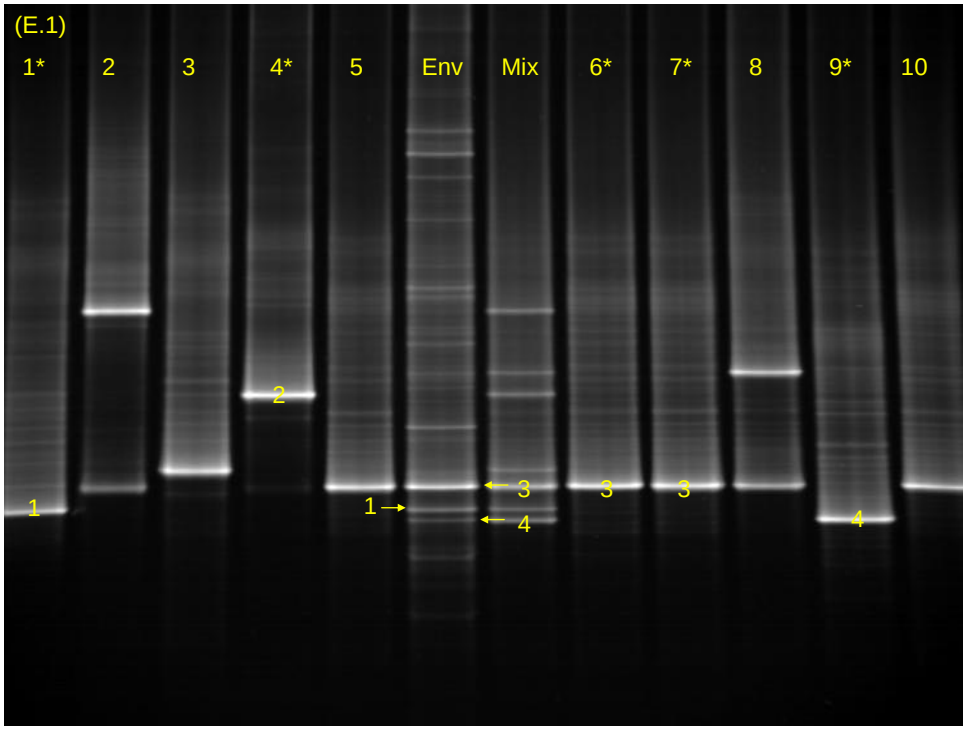


Figure A-17. TTWet 1-14-12 B Clones 1-10 (E.1). (**1**) Uncultured Ascomycota *(Articulospora tetracladia)* (**2**) Nematode DNA (**3**) *Penicillium sp.* (**4**) *Penicillium sp.*

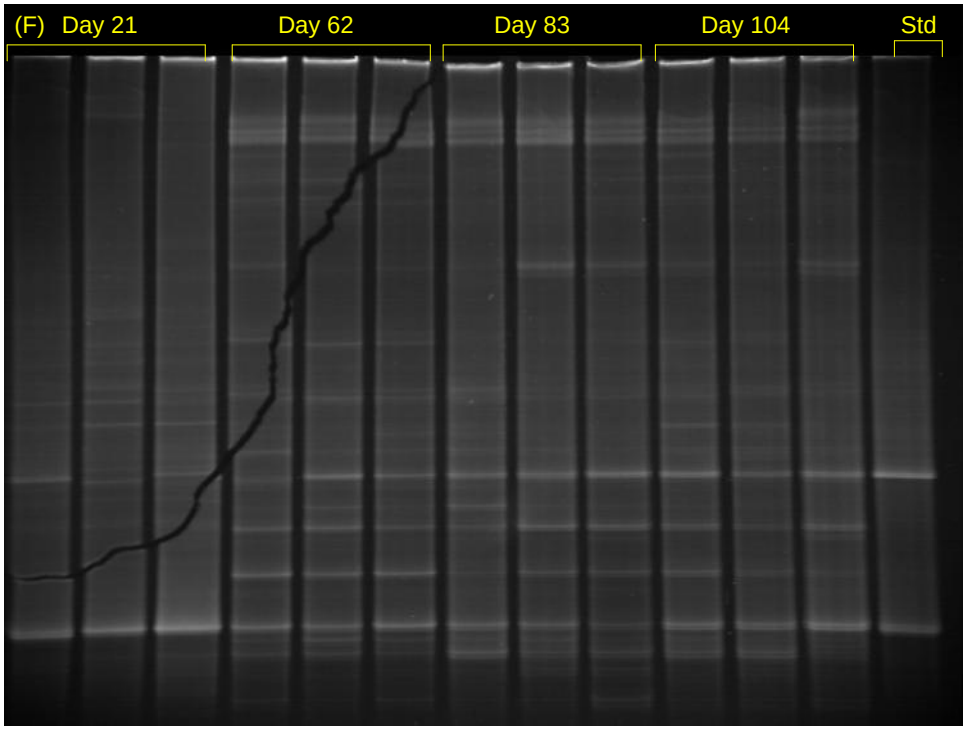


Figure A-18. BBDry Environmental DGGE (F).

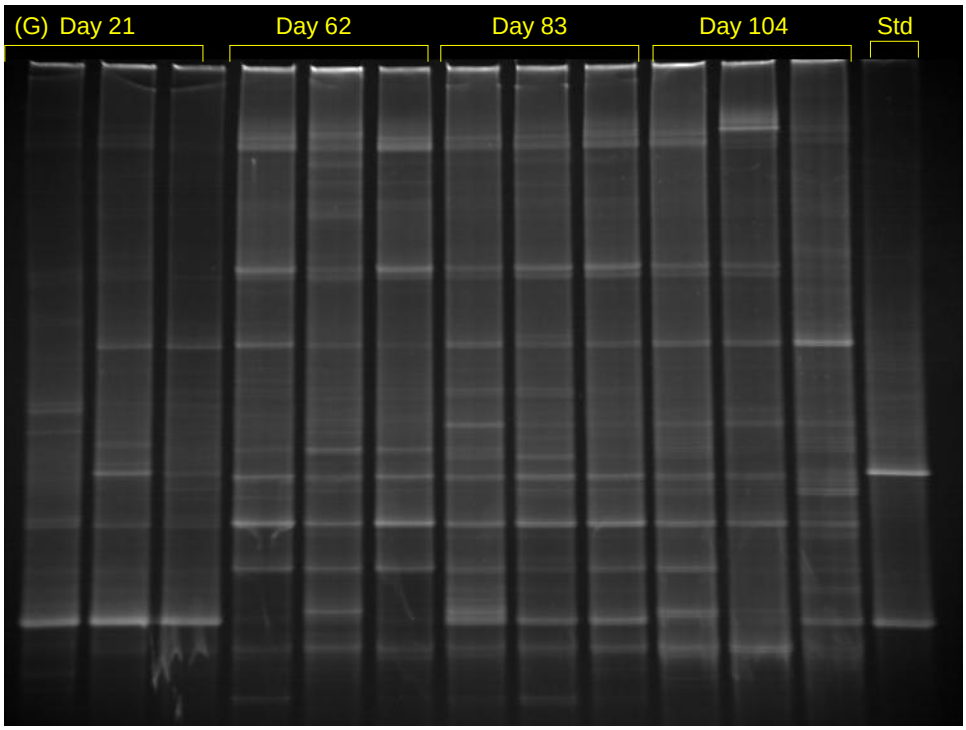


Figure A-19. BBWet Environmental DGGE (G).

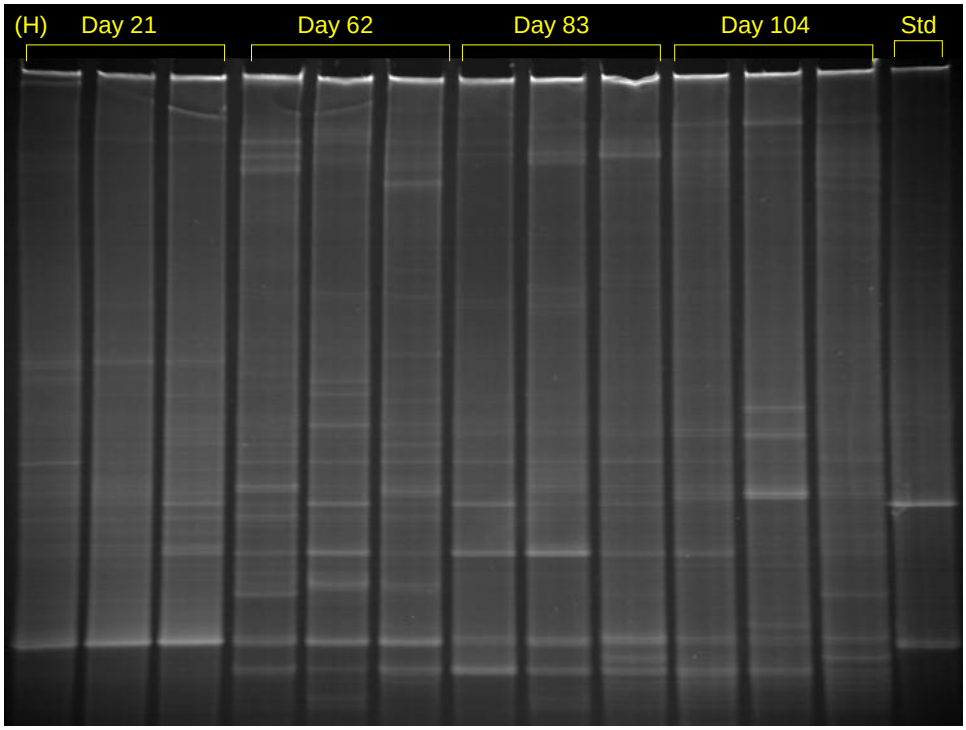


Figure A-20. KTDry Environmental DGGE (H).

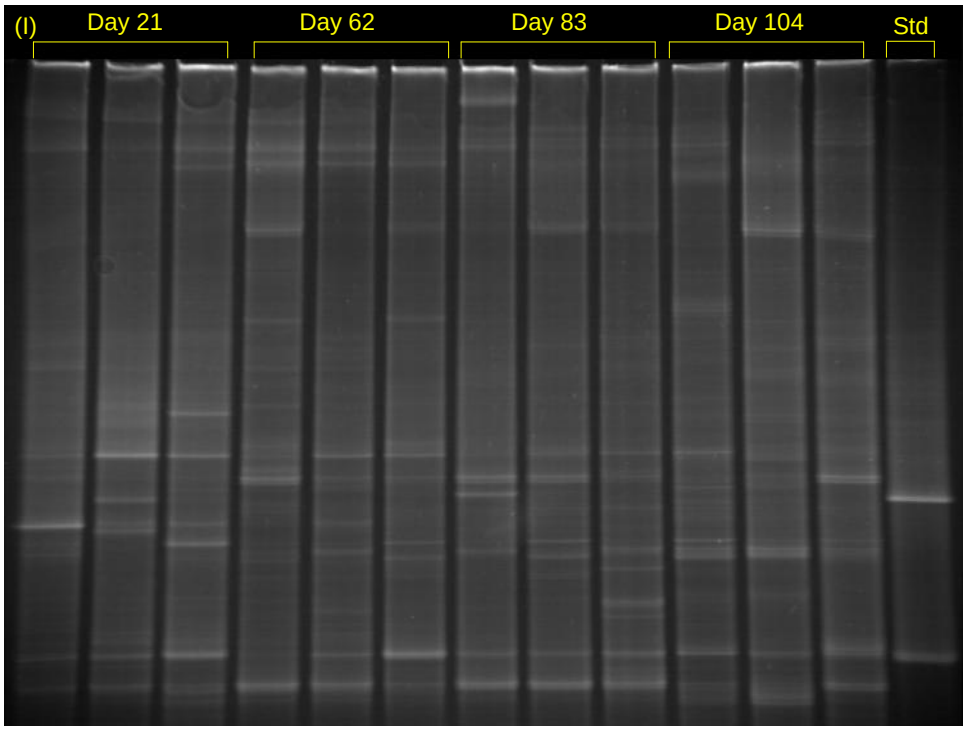


Figure A-21. KTWet Environmental DGGE (I).

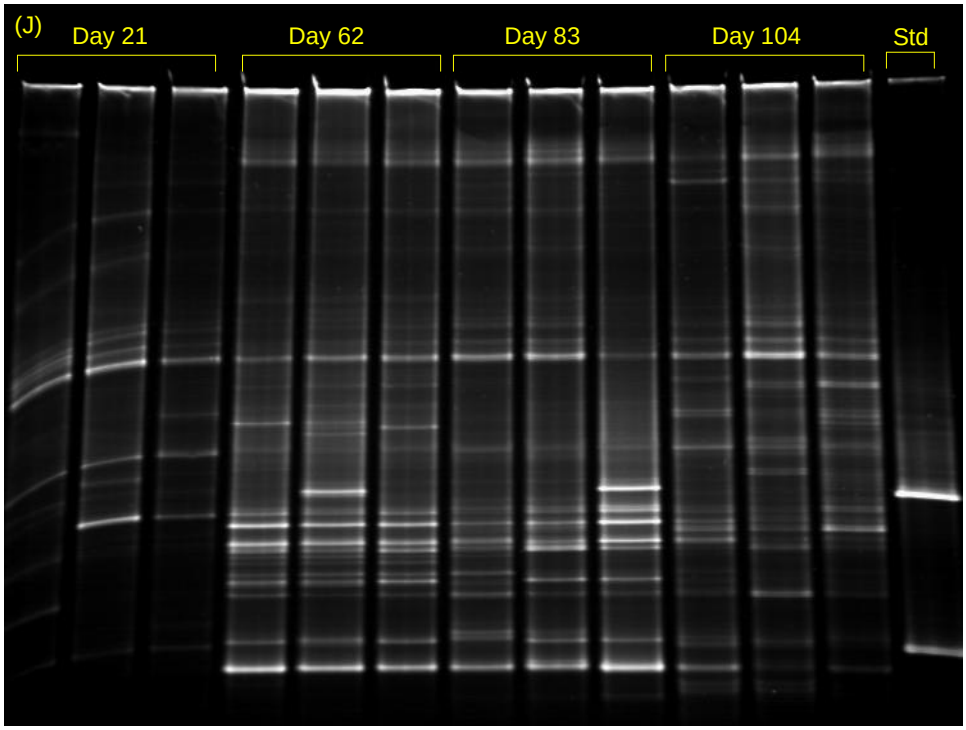


Figure A-22. QuarDry Environmental DGGE (J).

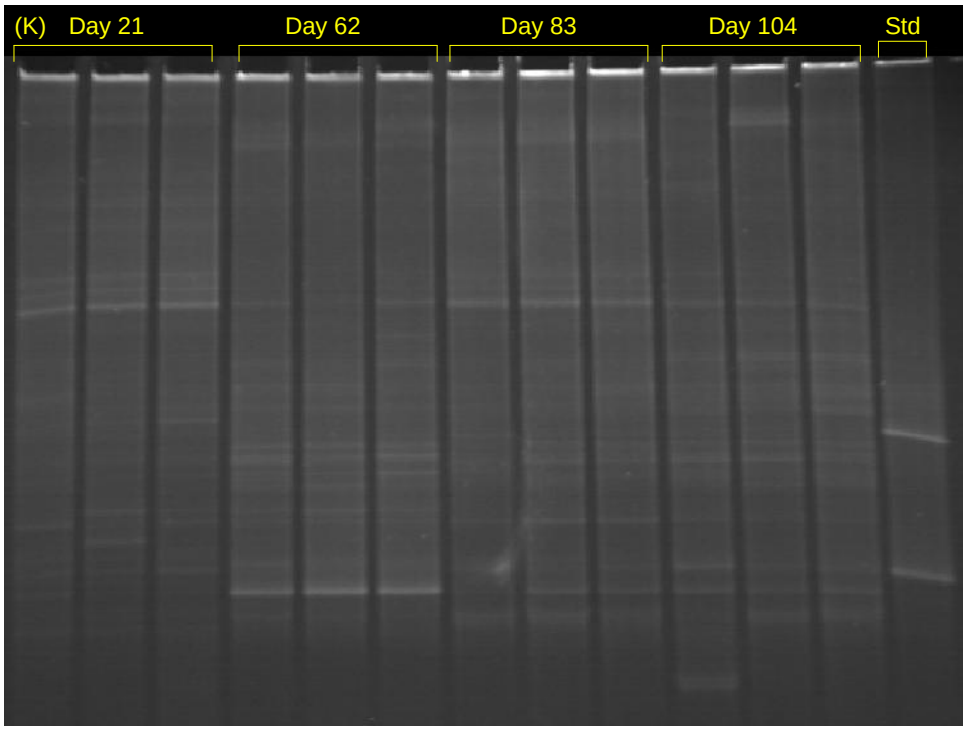


Figure A-23. QuarWet Environmental DGGE (K).

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VITA

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Having developed a love for teaching, caving, and science while attending Missouri S&T, he intends to pursue a Ph.D. in a biological field.