MICROBIAL COMMUNITY RESPONSES TO ENVIRONMENTAL CHANGE:
AN INVESTIGATION IN VERNAL POOLS

by

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Dedication

To my family. You have helped me turn my dreams into reality.
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Microbial Community Responses to Environmental Change: An Investigation in Vernal Pools

Abstract

by

SARAH ROSE CARRINO-KYKER

The effects of environmental perturbations on microbial community structure may strongly influence ecosystem functioning, as microorganisms play a dominant role in nutrient and carbon cycling. Vernal pools are an interesting model system for studying environmental effects on microbial structure and function, due to a physicochemical environment that is highly variable in space and time. In a field study, we found that changes in eukaryotic microbial communities of vernal pools were correlated with differences in a number of parameters, including dissolved oxygen, conductivity, urban land-use, and substrate quality. These results indicated that both landscape-level influences on vernal pool chemistry as well as local inputs of leaf litter are important for structuring microbial communities and further suggested that microbes may respond to multiple levels of environmental change.

In a microcosm study, we investigated the relationship between microbial community structure and function under different levels of environmental change: pH manipulation and nitrate (NO$_3^-$) addition. We found that bacterial and fungal communities were altered by pH, which corresponded with differences in respiration and suggested that broad changes can affect both structure and function. Denitrification, a microbial process which utilizes NO$_3^-$ as an electron acceptor, was not detected in the absence of NO$_3^-$ . This large change in function was independent of community structure
changes, as NO$_3^-$ addition had little effect on community structure of bacteria, fungi, or denitrifying organisms or on denitrifier relative abundance or population size, indicating that narrow changes may not affect community dynamics, even of functional groups. The microbial communities in the microcosms were further characterized with a metagenomic approach, which showed no difference in bacterial richness or diversity with NO$_3^-$ addition and reaffirmed previous conclusions that changes in denitrification could occur without altered microbial community structure. The metagenomes further revealed high metabolic diversity, suggesting that denitrifiers and other groups of vernal pool microbes may rely on physiological plasticity for growth when certain resources are limiting. Overall, our results suggest that microbial community stability and effects on function are highly context-dependent, driven by the nature of the environmental perturbation and the physiological plasticity of the communities affected.
Chapter 1
General Introduction and Conceptual Model

1.1 Microbial Ecology

1.1.1 Fundamental Reasons for Studying Microbial Communities

In a groundbreaking study, Woese and Fox (1977) used ribosomal RNA sequence analysis of representative organisms to determine the evolutionary relationships between all living systems. These authors found that a group of prokaryotes, at the time consisting solely of methanogenic bacteria, were equally different in their rRNA sequences from eukaryotes and from other prokaryotes. They argued that this group of methanogenic bacteria, which they called archaeabacteria, may represent a major group of organisms in addition to eukaryotes and other prokaryotes. In a subsequent publication, Woese et al. (1990) argued that the phylogenetic evidence collected between their 1977 study and 1990 suggested that living organisms are organized into three “domains” rather than five kingdoms. This restructuring of life on earth into the domains of Archaea, Bacteria, and Eukarya, the first two domains consisting solely of microscopic, single celled organisms, revealed that the majority of global diversity is predominantly, and perhaps overwhelmingly, prokaryotic (Pace, 1997; Amann, 2000). Studying this enormous diversity is imperative for understanding this group of organisms and how they impact our planet.

Studies on microbial richness, diversity, and community structure are important for a variety of ecological, educational, and technological reasons (Tiedje, 1994; Amann, 2000; Øvreås, 2000). Amann (2000) argued that since much of microbial diversity is still undiscovered, in-depth descriptions of microorganisms are necessary for microbiology to
reach the level of natural history that has been achieved in zoology and botany. Additional reasons for studying microbial ecology focus on the use of microbes as model systems for examining organismal survival strategies, patterns in the relative distribution of organisms, or regulations on biodiversity (Tiedje, 1994; Øvreås, 2000). Studies of microbial community structure can also lead to advances in biotechnology and the discovery of new pharmaceuticals (Tiedje, 1994; Øvreås, 2000). Finally, microbial community structure studies can be related to biogeochemical processes to infer environmental stability and long-term ecosystem function. This relationship between microbial community structure and ecosystem function is the subject of much current microbial ecology research and scientific debate (Zak et al. 2006).

Current studies in microbial ecology and biogeochemistry seek to link the community structure and function of microbial communities and explore how these relationships alter ecosystem processes (McGrady-Steed and Morin, 2000; Prosser, 2002; Heemsbergen et al., 2004; Deiglmayr et al., 2006; Wertz et al., 2006). Studies of this type are important because microbial communities exert a strong control on carbon and nutrient cycling in ecosystems, dominate decomposition processes, and either facilitate or comprise a large portion of system gross primary productivity. Therefore, changes in microbial community abundance, composition, or activity in response to environmental change could have dramatic and cascading impacts on long-term ecosystem function and stability. However, many past studies on the functional significance of microbial communities have measured process rates and pool size changes as a proxy for microbial function estimates, and this “black box” approach has avoided mechanistic linkage between microbial community composition and ecosystem processes (Prosser, 2002; Zak
et al., 2006). Making such connections is vital for understanding how ecosystems will respond to environmental changes (Balser et al., 2001; Wardle, 2002; Morris and Blackwood, 2007). Results from current studies on the relationship between community structure and ecosystem function are mixed. In aquatic microcosm experiments, McGrady-Steed et al. (1997) and Naeem and Li (1997) found positive relationships between microbial species richness and function (carbon dioxide flux and ecosystem reliability, respectively). However, Wertz et al. (2006) and Wertz et al. (2007) showed that soil function was maintained despite reduced microbial diversity. There is some evidence to suggest that the relationship between community structure and ecosystem function may be different for bacteria and eukaryotes, possibly due to differential responses of these communities to environmental variation (Marschner et al., 2003). Thus, there is currently no clear understanding of how altered microbial richness, diversity, or community structure, following environmental fluctuation, affects ecosystem processes. Additional studies on the relationship between microbial community structure and ecosystem processes are necessary for accurate prediction of ecosystem response to global change phenomena (e.g., climate warming, urban sprawl, etc.), which can lead to local extinctions of species.

1.1.2 Estimates of Microbial Diversity

It is difficult to make accurate estimates of prokaryotic diversity, mostly due to methodological problems. It has been estimated that only 1-2% of bacteria can be cultured with standard techniques (Amman et al., 1995; Prosser, 2002), and reliance on culture collections severely limits our perception of bacterial diversity. By extrapolating from the 1% of known bacterial species based on culturing and factoring in that
approximately 4500 species of bacteria and archaea have been described, Tiedje (1994) estimated that there are approximately 500,000 prokaryotic species worldwide. Other researchers have placed the estimated number of prokaryotic taxa (from the domains *Bacteria* and *Archaea*) in the millions (Trüper, 1992; Gans *et al.*, 2005), or in the billions (Dykhuisen, 1998) while Pace (1997) suggested that prokaryotic taxa are so numerous that an accurate number is unobtainable. Soil is perhaps the most diverse microbial environment. Dykhuisen (1998) estimated $10^9$ to $10^{12}$ bacterial species worldwide based on estimates of approximately half a million species in 30 grams of soil, and Gans *et al.* (2005) suggested that bacterial diversity can reach $10^7$ distinct taxa in just 10 grams of uncontaminated soil. These estimates exclude microbial eukaryotes, which include protists, unicellular algae, and microscopic fungal life stages, which are also important in a variety of ecosystems. Studying this extraordinary diversity can, at times, be a daunting task. This is especially true given the fact that there is debate within the field of microbiology as to what actually constitutes a bacterial species and what is the best species concept for microorganisms and, in particular, for prokaryotes (reviewed by Rosselló-Mora and Amann, 2001).

Currently a prokaryotic species is defined as a collection of individuals which have a variety of major properties in common, but which differ significantly from other groups in one or more of these properties (Madigan *et al.*, 2003). The cellular properties used to define a prokaryotic species include DNA:DNA hybridization, thermal denaturation, and 16S rRNA gene sequence similarity above a certain threshold value (McArthur, 2006). These criteria for defining a prokaryotic species, which rely heavily on laboratory techniques and have evolved as new methods are developed, have been
criticized as being too conservative (Dykhuisen, 1998; Whitman et al., 1998). Thus, a variety of new concepts have been developed. For example, the phenetic species concept, which is most closely related to the current way in which prokaryotic species are defined, involves classifying organisms based on a variety of morphological, physiological, and molecular characteristics. These include general morphology (cocci, spirochete, rod, etc.), growth on various organic compounds, nitrogen source use, pH range, DNA:DNA hybridizations, and the guanine/cytosine content (McArthur, 2006). Alternatively, the phylogenetic species concept does not rely so heavily on laboratory techniques and, instead, involves observations of the fossil record in combination with molecular sequencing (McArthur, 2006). Because prokaryotic fossils are exceedingly rare, molecular data have been used to infer evolutionary relationships and distinguish between species based on the phylogenetic species concept. Rosselló-Mora and Amann (2001) have proposed the phylo-phenetic species concept, which incorporates aspects of both the phenetic and phylogenetic species concepts. Currently there are as many as 20 prokaryotic species concepts, the description of which is beyond the scope of this dissertation. However, as studies of microbial community structure continue, it is important to reach some level of consensus in order to accurately determine prokaryotic diversity. Otherwise, there is a risk of underestimating or overestimating the true diversity of organisms in the domains Bacteria and Archaea (Dykhuizen, 1998).

1.1.3 Current Methods in Microbial Ecology

Enormous progress has been made in the last 20 years in understanding the structure of prokaryotic communities and expanding the knowledge of eukaryotic microbial communities (e.g., micro-algae and microscopic life stages of fungi), mostly due to the
development of DNA-based methods and their application to microbiological studies (Amann et al., 1995; Head et al., 1998; Amann, 2000; Øvreås, 2000, Dahllöf, 2002). Many environmental genomic studies have focused on 16S or 18S rRNA gene sequences because of their ubiquity in all species and general acceptance for revealing both taxonomic and evolutionary relationships. These studies have vastly increased knowledge of microbial community structure by identifying sequences from organisms which cannot be cultured. For example, the construction of clone libraries from DNA of prokaryotic picoplankton and small eukaryotic plankton has revealed high levels of diversity and new groups of organisms in marine habitats (Giovannoni et al., 1990; Fuhrman et al., 1992; DeLong et al., 1994; López-García et al., 2001).

Molecular techniques have allowed exploration of ecological patterns in microbial communities which have traditionally been limited to macroscopic organisms, such as the taxa-area relationship (Horner-Devine et al., 2004) the productivity-diversity relationship (Horner-Devine et al., 2003), and comparisons of community structure across environmental gradients (Lüdemann et al., 2000; Norris et al., 2002; Rothrock and Garcia-Pichel, 2005) and between samples from different environmental conditions (van Hannen et al., 1999; Schäfer et al., 2001; Nikolcheva et al.; 2005; Burke et al., 2008). Techniques such as denaturing gradient gel electrophoresis, restriction fragment length polymorphism, and terminal restriction fragment length polymorphism have all been used to describe microbial community patterns. All of these techniques (i.e., cloning and profiling techniques) rely on amplification of target genes through polymerase chain reaction (PCR) and consequently can be biased both by PCR conditions and primer specificity (see Head et al., 1998 and Kanagawa, 2003 for a complete review).
Consequently, all of these profiling techniques probably underestimate microbial diversity and fail to provide sufficient data relating to the abundance of these organisms in the environment. Nevertheless, these techniques are some of the most powerful tools currently available for exploring microbial community structure in natural systems.

Of equal importance to exploring the factors influencing microbial community structure are studies which explore how these communities function. By using molecular methods to target functional genes, the richness, diversity, and structure of different functional groups can be determined (Wawer et al., 1997; Alfreider et al., 2002; Burke et al., 2002; Deiglmayr et al., 2006;). Quantitative PCR (QPCR) can be used to supplement profiling techniques to determine gene copy numbers in natural environments (López-Gutiérrez et al., 2004; Kandeler et al., 2006; Mosier and Francis, 2008). When targeting DNA, QPCR can be used to estimate microbial population size when functional genes are targeted (Rinta-Kanto et al., 2005; Kandeler et al., 2006; Mosier and Francis, 2008). In addition, when mRNA is targeted, QPCR can provide information about gene expression and microbial activity. Of great promise are metagenomic studies which amplify regions throughout the genomes of mixed microbial communities for sequencing. In this way, both phylogenetic and functional diversity of communities can be obtained (Venter et al., 2004; Tringe et al., 2005; Edwards et al., 2006; Dinsdale et al., 2008). All of these techniques permit examination of microbial community structure and functional activity in natural environments and allow exploration of fundamental questions in microbial ecology.

1.2 Microorganisms in a Dynamic Environment

1.2.1 Environmental Controls on Microbial Growth
Microorganisms survive and grow in a wide array of environmental conditions, from the high temperatures of hot springs (Ferris *et al.*, 1996) to the freezing temperatures of Antarctic ice (DeLong *et al.*, 1994), as well as other extreme environmental conditions found in deep ocean vents (González *et al.*, 1995; Byrne *et al.*, 2009) or associated with acidic mines (Edwards *et al.*, 2006; López-Rodas *et al.*, 2008). That microbes can thrive in so many different environmental conditions is phenomenal given that, due to their small size, they are incredibly sensitive to environmental fluctuations (McArthur, 2006). The presence of diverse groups of microorganisms in so many environments is a consequence of evolved morphological and biochemical adaptations which enable these organisms to perform the cellular functions necessary for their survival, growth, and reproduction even in extreme environments. Individual microbes have environmental ranges within which they can maintain homeostasis despite fluctuating environmental conditions; however, as environmental conditions approach the physiological limits of a microbe, they become less efficient and eventually can no longer survive (Madigan *et al.*, 2003). There are four environmental parameters which are considered major controlling factors on microbial growth: oxygen, temperature, water availability, and pH (Madigan *et al.*, 2003). These four factors are a simplification of the many environmental changes that can influence microbial growth, but because these factors affect the biochemical processes of microbial cells and are integrative measures of other environmental changes, they can strongly influence the microbial species which can survive and function in a given environment (see below).

1.2.1a Environmental Controls: Oxygen
Microorganisms can use a variety of terminal electron acceptors for cellular respiration, and the presence of these electron acceptors often determines the spatial distribution and abundance of soil microbes. Many microorganisms are obligate aerobes and require oxygen for cellular respiration, whereas microaerophilic organisms require oxygen but only at low concentrations. Along vertical soil gradients, oxygen concentration is one of several parameters that decrease with soil depth. For example, Lüdemann et al., 2000 found a markedly different bacterial community composition in shallow versus deeper soil layers and suggested that this was due to differing oxygen concentration. Since oxygen is weakly soluble in water, water-saturated environments, such as many wetland soils and sediments, have low concentrations of oxygen which limit the growth of aerophilic organisms. Hence, bacterial and archaeal taxa that have evolved alternative respiratory pathways predominate under these conditions. Examples of alternative terminal electron acceptors in the absence of oxygen include nitrogenous compounds (Knowles, 1982; Burgin and Hamilton, 2007), sulphur-containing compounds (Colleran et al., 1995), iron or manganese-containing compounds (Lovely and Phillips, 1988; Myers and Nealson, 1988), and humic substances (Scott et al., 1998). These alternative respiratory pathways are essential for ecosystem energy and nutrient cycling.

1.2.1b Environmental Controls: Temperature

Microorganisms are often traditionally grouped by the range of temperatures in which they can grow and survive (Madigan et al., 2003; McArthur, 2006). Traditional groupings of microbes by temperature include 1) psychrophiles, which thrive in cold temperatures (15°C or colder) typical of polar regions; 2) mesophiles, which are adapted to temperatures between 15°C and 45°C and inhabit the digestive tracts of warm-blooded
animals and temperate and tropical forests and lakes; and 3), thermophiles and hyperthermophiles, which have temperature optima between 45°C and 80°C and above 80°C, respectively, and are found in hot springs, geysers, and deep-sea hydrothermal vents. Temperature is important for the growth and survival of microorganisms because it affects the enzyme kinetics of a cell and the stability of the cell’s cytoplasmic membrane. Psychrophiles possess enzymes with greater amounts of α-helices and lesser amounts of β-sheets for secondary structure, because the latter form a more rigid structure which makes the enzyme less flexible in the cold (Madigan et al., 2003). Psychrophiles also contain greater amounts of unsaturated fatty acids in their cytoplasmic membranes to keep the membranes fluid in cold temperatures (Madigan et al., 2003). In contrast, membranes of thermophilic microbes typically contain saturated fatty acids, which confer greater stability to the membranes at high temperatures, and many of the enzymes of these microbes can contain one of a few critical amino acid substitutions or a greater number of ionic bonds between amino acids to facilitate heat stability (Madigan et al., 2003). In addition, thermophiles and hyperthermophiles produce a variety of compatible solutes which likely help with protein stability at high temperatures (Kanodia and Robers, 1983; Scholz et al., 1992; Martins and Santos, 1995; Martins et al., 1997; Lamosa et al., 1998). Although these adaptations allow microbes to survive in extreme environments, microbial community structure can vary across even small gradients of temperature (Chin et al., 1999; Norris et al., 2002), which highlights the importance of temperature for microbial distribution.

1.2.1c Environmental Controls: Water
Like all other organisms, microorganisms require water for growth. Thus, water content in an environment can be a strong determinant of microbial community structure (e.g., Drenovsky et al., 2004). Microbes also vary in their tolerance to dry environments. For example, along a desiccation gradient in intertidal zones (Rothrock and Garcia-Pichel, 2005) and over time as a temporary pond dried (Bärlocher et al., 1978), the microbial community shifted as water became scarcer. Some organisms, called xerophiles are capable of surviving in very dry environments, such as the arid soils common to deserts and cinder fields (Dunbar et al., 1999; Fierer and Jackson, 2006). Other organisms, called halophiles, thrive in high salt environments, such as the Great Salt Lake (Post, 2005). These organisms prevent loss of water via osmosis by increasing their internal solute concentration. These compatible solutes can be amino acids, carbohydrates, alcohols, or of other chemical structure (Madigan et al., 2003).

1.2.2 The Effect of pH on Microorganisms

Recently, there has been increased interest in the effect of pH on microorganisms in natural environments. This interest is partly the result of a study by Fierer and Jackson (2006), in which 98 soil samples from an array of distinct locations (classified into six different ecosystems) across North and South America were sampled and bacterial community structure in these soils was correlated with environmental characteristics. Of all the soil factors examined in this global-scale study, pH was found to be the best predictor of bacterial diversity and richness. Though pH seems like a relatively crude environmental parameter, pH is actually a useful integrative metric of chemical conditions experienced by organisms, because the concentration of $H^+$ ions influences
both biochemical processes of individual cells (McArthur, 2006) and biogeochemical processes in the surrounding environment (Stumm and Morgan, 1996).

Microbes have an optimal pH range, typically a maximum of 2-3 units wide, within which the biochemical processes necessary for respiration and growth are possible (McArthur, 2006, Madigan et al., 2003). The majority of microbes grow between pH 2 and 10, and only a few extremeophiles can survive below or above this range (Madigan et al., 2003). Biochemical adaptations which maintain cytoplasmic pH at or near its optimum are critical for cell survival, especially in extreme pH environments (Kushner, 1993). Many studies have shown that internal pH may be regulated by active ion transport across the cytoplasmic membranes of the microbial cells (reviewed by Booth, 1985), but other studies indicate that pH maintenance may be a passive process (i.e., due to cellular adaptations and not active processes), such as the presence of charged amino acids in the cell to buffer the cytoplasm or less permeable membranes to prevent proton diffusion (McArthur, 2006). Maintenance of cell pH is necessary for two critical reasons: 1) maintenance of the protonmotive force and 2) maintenance of internal cellular activity.

Across cellular membranes is an electrochemical gradient created by the transfer of electrons from inside the cell to outside via passive diffusion, active transport, and ATP hydrolysis (McArthur, 2006). This gradient is referred to as the protonmotive force and has two components: a pH gradient (ΔpH) with more acidic conditions outside and a membrane potential (ΔΨ) with positive conditions outside (McArthur, 2006). One important use of the protonmotive force is that it allows for the active transport of materials, such as nutrients, into the cell from the environment. This cotransport, where protons which have been pumped outside the cell diffuse back inside through membrane
proteins called cotransporters, and amino acids, sugars, or other nutrients are pumped into the cell along with the proton, is an essential uptake mechanism for cellular survival. (See Figure 1.1, protonmotive force panel.)

Microorganisms which live in extreme pH environments have evolved strategies to maintain their protonmotive force under highly acidic or basic conditions (i.e., conditions in which the external proton concentration is high or low, respectively). In highly acidic environments, where the microorganisms must deal with high proton concentrations outside the cell, acidophilic microbes reverse their membrane potential such that the inside of the cell is positive (Booth, 1985). This allows the cells to limit the amount of protons that would normally diffuse inside the cell down the electrochemical gradient, and thereby regulate the level of compounds which can enter the cell and keep the internal pH stable. In contrast, alkalophilic microbes have a very high positive ΔΨ outside the cell to allow protons, which are in very low concentration externally, to continue to be pumped from the outside to the inside of the cell (Booth, 1985). However, the environmental pH does affect internal pH, which means that microbes adapted to a certain pH range must also be able to maintain their internal cellular activity, such as enzyme function, at a potentially non-neutral pH (Booth, 1985; Lowe et al., 1993; see Figure 1.1 enzyme activity panel). This is especially true for anaerobic microorganisms which are less likely to use energy to maintain their proton motive force, because they derive less energy from their terminal electron acceptors than aerobes do from using oxygen (Lowe et al., 1993). Thus, the microbial species which can survive at a given pH are those which can maintain both their protonmotive force and internal cellular activity. These biochemical limitations, then, are likely to result in a different microbial
community composition as a function of changes in environmental pH simply because not all microbes have biochemical adaptations which permit growth at every pH level (Figure 1.1).

The pH (an approximation of the H\(^+\) concentration) of environments also affects chemical processes which are involved in the formation, alteration, and dissolution of minerals (Stumm and Morgan, 1996). Since many chemical species which are affected by pH are also potential electron donors or electron acceptors for microbes, alterations in the oxidation state and availability of chemical species and as a function of environmental pH can have cascading effects on the composition of the microbial community (Figure 1.1, resource availability panel). Ideal habitats within which to test the effects of environmental conditions on microbial diversity and function are those in which abiotic parameters fluctuate spatially and temporally. Vernal pools are examples of such habitats.

1.2 The Microorganisms of Vernal Pools

In the temperate regions of North America, seasonally flooded ponds, called vernal pools, are found throughout the landscape during the spring season. Vernal pools are commonly known as sites of amphibian breeding and, as such, many studies on vernal pool ecology have focused on frogs or salamanders (e.g., Thompson and Gates, 1982; Seale, 1982; Dale et al., 1984; Freda and Dunson, 1985; Freda and Dunson, 1986; Taylor et al., 1988; Sadinski and Dunson, 1992; Laposata and Dunson, 1998; DiMauro and Hunter, 2002; Skelly et al., 2002). Much less attention has been given to the microbial communities of vernal pools even though these communities underlie ecosystem element cycling and energy flow in both terrestrial and aquatic habitats (Etherington, 1975; Mitsch and
Gosselink, 1993; Stumm and Morgan, 1996). In vernal pools, the importance of microorganisms for element cycling and energy flow has been indicated by studies that have shown considerable allochthonous leaf litter input and high decomposition rates (Bärlocher et al., 1978) and a richness of microbial groups which are instrumental in decomposition (Carrino-Kyker and Swanson, 2008).

Vernal pools are spatially and temporally dynamic in terms of their physical and chemical conditions. Environmental parameters, such as pH, water temperature, dissolved oxygen, and depth, fluctuate widely and rapidly over the hydroperiod of these shallow, temporary ponds (Bonner et al., 1997; Carrino-Kyker and Swanson, 2007), and these fluctuations contribute to a temporally dynamic microbial community (Carrino-Kyker and Swanson, 2008). In other aquatic, marine, and terrestrial habitats, many studies have documented changes in microbial community composition with shifts in environmental conditions (e.g., van Hannen et al., 1999; Burke et al., 2002; Dorigo et al., 2002; Rothrock and Garcia-Pichel, 2005; Burke et al., 2008). Recent studies have also shown correlations between microbial communities and ecosystem function (as described in section 1.1.2). However, few studies have documented linkages between microbial communities and environmental conditions in vernal pools (Carrino-Kyker and Swanson, 2008), and, to my knowledge, no studies have attempted to correlate community change with ecosystem function in these habitats. Thus, my dissertation research has utilized vernal pool habitats to explore the diversity of microbial communities and the relationship between microbial diversity and function.

1.3 Conceptual Model and Overall Purpose
In work for my master thesis, we found high levels of spatial and temporal variability, in environmental conditions for 35 vernal pools of Northeastern Ohio. In particular, we found pH to vary by 3-4 pH units throughout the spring season in these vernal pools (Carrino-Kyker and Swanson, 2007; Carrino-Kyker and Swanson, 2008). In a subset of five pools for which both bacterial and eukaryotic microbial community structure was studied over time, changes in the composition of both communities was strongly correlated with changing pH (Carrino-Kyker and Swanson, 2008). The mechanisms for this pH effect on microbes are likely both biochemical and biogeochemical (described above in section 1.2.2). Thus, I propose that the realized microbial community at a given pH passes through two environmental filters: 1) a biochemical filter, where surviving microbes can maintain their protonmotive force and cytoplasmic cellular functions and 2) a biogeochemical filter, where the surviving microbes are still able to perform processes dependent on the availability of chemical compounds (Figure 1.1). The strengths of these filters can affect microbial community composition and, potentially, ecosystem processes (Figure 1.1).

This relationship between microbial community structure and ecosystem processes may, however, be context-dependent. When environmental changes affect broad physicochemical conditions (“modulators” after Balser et al., 2001), species diversity and community structure may change, but long-term ecosystem function should be unaffected as a consequence of high functional redundancy within the system. On the other hand, environmental changes which alter the availability of chemical compounds utilized by functional groups (or “resources” after Balser et al., 2001) should affect specific microbial functional groups and, therefore, should be accompanied by changes in
the ecosystem processes which these functional groups perform (Balser et al., 2001). The Balser et al. (2001) paradigm suggests that changes in modulators, such as altered pH or an increase in temperature, should correspond with broad changes in microbial community structure with no long-term affect on ecosystem functions. In other words, though there may be compositional instability following a modulator change, because many different microbial species are capable of performing the same ecosystem processes, such as respiration and carbon cycling, these functions are predicted to remain constant even though there may be species turnover (Balser et al., 2001; Figure 1.1, yellow arrow). On the contrary, changes in resources, such as an increase in nitrate associated with nitrogen enrichment, should lead to altered community structure in microbial functional groups as well as the functions these groups perform, such as denitrification (Balser et al., 2001; Figure 1.1 blue arrows). This model proposed by Balser et al. (2001) is an interesting framework within which to study the relationship between microbial diversity and ecosystem function. However, the model does not address the fact that environmental parameters are often intertwined. Thus, I have further hypothesized that changes in some modulators, such as pH, can interact with changes in specific resource limitation (e.g., biochemical changes in resource availability) to alter system function beyond that predicted from either alone (Figure 1.1 green arrow). Grounded in these hypotheses, my dissertation research addresses the following questions:

1. *How is the microbial community structure altered by large landscape-level environmental variability?*

2. *What is the relationship between microbial community structure/diversity and the functional attributes of vernal pools and is this relationship context-dependent?*
3. Can current technologies, which provide a more accurate representation of microbial taxonomic and functional diversity than the commonly used profiling techniques, provide a clear relationship between microbial community structure and ecosystem function?

This dissertation contains three separate but interrelated studies with the overall objective to describe how environmental fluctuations influence microbial community structure and how changes in microbial richness, diversity, and composition, in turn, affect ecosystem function. The research described herein is a continuation of my master’s thesis, which involved a descriptive characterization of abiotic conditions and microbial communities of vernal pools in Northeastern Ohio (Carrino-Kyker and Swanson, 2007; Carrino-Kyker and Swanson, 2008). My Ph.D. research began with a survey of eukaryotic microbial communities in vernal pools at a landscape scale, in which the eukaryotic microbial community was assessed for spatial variation and correlated with environmental variability and human land use across the landscape (Chapter 2). With the new hypotheses that emerged from this descriptive study and my master’s work, we constructed a manipulative microcosm experiment, in which pH and nitrate concentration were manipulated (Chapter 3). In this microcosm experiment, the microbial functions of denitrification, respiration, and decomposition were measured, and these processes were correlated with microbial community structure, specifically bacteria, fungi, and denitrifying bacteria. Finally, an in-depth DNA sequencing analysis, which included functional genes, was conducted to gain a more accurate understanding of microbial and functional diversity in this understudied habitat (Chapter 4).
Figure 1.1 Conceptual model on how environmental changes affect microbial community structure and function. With a change in environmental pH, the realized microbial community is predicted to pass through two filters: a biochemical filter and a biogeochemical filter. Changes in microbial community structure, then, could impact ecosystem function, especially processes performed by functional groups (see text for details).
Chapter 2

Changes in Eukaryotic Microbial Communities of Vernal Pools along an Urban-Rural Land Use Gradient

2.1 Summary

Seasonally flooded vernal pools are common throughout temperate forest landscapes and may represent significant sites of forest nutrient cycling. The effects of urbanization on vernal pool microbial communities, important drivers of nutrient cycling, are largely unknown; thus studies to improve our understanding of microbes and their functional roles in these habitats are needed. Eukaryotic microbial communities were sampled from 30 vernal pools of the Cuyahoga River watershed, located along a gradient of urban land use, were profiled with denaturing gradient gel electrophoresis, and were compared between pools using nonmetric multidimensional scaling (NMS). Microbial richness, and, specifically, the richness and diversity of the fungal operational taxonomic units (OTUs), increased with urbanization. Vernal pool eukaryotic microorganisms formed two NMS clusters that differed significantly in sub-watershed urban area. However, the significance of urbanization disappeared when fungal and algal communities were analyzed separately. Water conductivity was consistently correlated with different microbial communities (e.g. eukaryotic, fungal, and algal). Fungal communities also appeared related to the carbon content of the substrate, indicating that vegetation at a local scale may be important for community structure. Almost half of the OTUs matched fungal species, which provides taxonomic evidence that the eukaryotic microbial communities of vernal pools are dominated by fungal species. Overall, our data suggest
that the eukaryotic microbes of vernal pools are influenced by a variety of factors of the surrounding landscape, including urbanization, water chemistry, and vegetation type.

2.2 Introduction

In the temperate regions of North America, seasonally flooded ponds, called vernal pools, are found throughout the landscape during the spring. Similarly, vernal pools can be found on a variety of soil types, underlying geologies, and surrounding land uses (reviewed by Colburn, 2004). For example, in a study of 106 vernal pools found in Amherst, Massachusetts, Stone (1992) found that these pools were distributed on soil from seven different drainage classes and of six different origins. Such differences in underlying material, accompanied with the variety of other factors that can change across the landscape, can lead to spatially variable water chemistry in vernal pools. In a study of 30 vernal pools of Northeastern Ohio, Carrino-Kyker and Swanson (2007) found correlations between conductivity and the degree of agricultural land use and between water depth and the degree of urban area that surrounded the pools. In a smaller study of four vernal pools in Massachusetts, Brooks et al. (2002) also found an effect of urbanization on water chemistry. Specifically, these authors noted higher temperature, pH, alkalinity, and conductivity in urban pools compared to non-urban pools. Such changes in the physical and chemical environment can affect members of the biological community, such as amphibians whose larval survival can be influenced by water chemistry (e.g., Freda and Dunson, 1985; Laposata and Dunson, 1998), insects whose development is strongly tied to the length of the hydroperiod (Brooks, 2000), or microorganisms, which are perhaps the most environmentally-sensitive organisms due to
their small size (McArthur, 2006). This latter group of organisms has been particularly understudied in vernal pools.

Vernal pools are commonly known as sites of amphibian breeding and, as such, many vernal pool studies have focused on frogs or salamanders (reviewed in Colburn, 2004). Much less attention has been given to the microbial communities of vernal pools even though these communities are instrumental in ecosystem element cycling and energy flow in both terrestrial and aquatic habitats (Bing-Ru et al., 2006; Gutknecht et al., 2006). Vernal pools may represent “hot spots” of nutrient cycling within the forest due to microbial processing of 1) allochthonous leaf litter inputs, which are colonized by microbes in both the dry and wet phases and 2) the excrement and debris (e.g., exoskeletons) left behind by aquatic organisms that live in the pool (Williams, 2006).

The microorganisms of vernal pools, then, comprise the base of the detrital-based food web upon which more complex organisms rely. Importantly, we do not have a clear understanding of what major taxonomic groups of microorganisms are present in these habitats, how the diversity of these communities is affected by water chemistry or urbanization, or the functional significance of these organisms in vernal pools. Carrino-Kyker and Swanson (2008) conducted a study of five vernal pools over three months and determined that both the bacterial and microbial eukaryotic communities changed rapidly in time, possibly as a consequence of changes in water chemistry and dominant abiotic conditions. This previous study, however, was done on a small number of pools at only one site; thus, the effects of landscape level influences, such as urbanization and associated water chemistry changes, on these communities are largely unknown. Further, previous studies of vernal pool microbial communities were completed prior to the
development of environmental genomic technologies (e.g., Bärlocher et al., 1978; Laird, 1988). In the current study, we examined the eukaryotic microbial communities in 30 vernal pools within a landscape of mixed land use types and utilized an urban-rural gradient to explore effects of urbanization on microbial communities in these pools (see Carrino-Kyker and Swanson, 2007). Such urban-rural gradients are interesting settings to ask basic ecological questions, test theories, and contribute to ecosystem management (McDonnell and Pickett, 1990). Our study specifically addressed whether the eukaryotic microbial community was altered by changes in watershed land use, because there is conflicting evidence on whether urbanization affects the richness and diversity of eukaryotic microorganisms negatively (Jumpponen and Jones, 2009), positively (Dopheide et al., 2009), or has no effect (Cousins, 2003; Duong et al., 2007). We also sought to determine if and how the composition of the eukaryotic microbial community was affected by urbanization. The eukaryotic microbial community was profiled using denaturing gradient gel electrophoresis (DGGE) and sequence analysis of excised bands, which allowed for more accurate estimations of diversity, community composition, and identification of major taxonomic groups than has previously been possible.

2.3 Materials and Methods

2.3.1 Field Sampling and Environmental Parameters

In March of 2004, 30 vernal pools in the Cuyahoga River Basin (near Cleveland, OH, USA) were sampled. The pools were located in ten sub-watersheds (n =3 pools per sub-watershed), which are natural boundaries defined by the water flow direction and accumulation of tributaries in the Cuyahoga River Basin, and all were found in deciduous forest regions. The percentage of each sub-watershed that represented a certain land use
category was determined with digital maps (W.B. Clapham, Jr., personal communication) and GIS (Figure 2.1). The ten sub-watersheds used in this study were selected to represent a gradient of urbanization (quantified as the sum of percent urban, percent suburban, and percent barren, which were mostly construction sites), but agricultural and natural land use classifications were also prevalent (Figure 2.1). A more thorough description of how sub-watersheds were delineated and how land use was assessed can be found elsewhere (Carrino-Kyker and Swanson, 2007). From each pool, three different sample types were taken 1-m from the edge of the pool. Soil cores (taken with cut, sterile pipet tips), leaves, and water were sampled in triplicate and combined for microbial community analysis. Once in the lab, 50 ml of the combined water sample were filtered through 0.2-µm polycarbonate filters to collect the organisms. The environmental parameters of pH, dissolved oxygen, temperature, and depth were measured *in situ*. Conductivity was determined on water samples that had been transported to the lab on ice no longer than seven hours. Specific descriptions of these measurements are described in Carrino-Kyker and Swanson (2007). We also analyzed representative leaf samples for %Carbon, %Nitrogen, and C:N with an elemental combustion system (Costech Analytical Technologies, Inc., Valencia, CA).

### 2.3.2 DNA Extraction and PCR

DNA was extracted from each sample type (soil, leaf, and filtered water) using the Fast DNA® Spin Kit for Soil (MP Biomedicals LLC, Solon, OH) and following the manufacturer’s protocol with the modifications described in Carrino-Kyker and Swanson (2008). Following the extractions, DNA from the three sample types was combined for each vernal pool (i.e., DNA was a composite of soil, litter and water). Eukaryotic
community DNA was then PCR-amplified from these combined DNA samples using the
primers and temperature cycling outlined by van Hannen et al. (1999), except that the
final extension step was 30 min. to eliminate DGGE double banding (Janse et al., 2004).
The forward primer contained a GC clamp on the 5’ end (van Hannen et al., 1999). For
each vernal pool sample, four DNA dilutions (1:5, 1:10, 1:20, and 1:40) were used in
PCR reactions to limit bias (Chandler et al., 1997). The DNA concentration in each
reaction, then, ranged from 0.5 ng and 14.0 ng between the 1:40 and 1:5 dilutions,
respectively. The PCR reactions were 50µl in volume containing 0.5 µM of each primer,
200 µM each dNTP, 1.5 mM MgSO₄, and 1 unit of Platinum® Taq DNA Polymerase
High Fidelity (Invitrogen Inc., Chicago, IL) for an automatic “hot start” to minimize
nonspecific binding of the primers to non-target DNA. Amplifications were performed

2.3.3 DGGE and Band Profiling
Positive PCR reactions (of the four dilutions) for each vernal pool sample were combined
equally to total 32 µl for each vernal pool. These combined PCR products were
subsequently loaded onto DGGE gels such that the concentrations ranged from
approximately 0.4 to 2.0 µg of PCR product for each vernal pool. Different DNA
sequences present in this combined PCR sample were separated on 1 mm thick DGGE
gels as described by van Hannen et al. (1999). We included a 2 cm stacking gel which
contained no denaturants through which samples were electrophoresed at 150 volts.
Bands were visualized by staining with a 1:10,000 dilution of SYBR gold (Molecular
Probes, Eugene, OR) in 1X Tris Acetate EDTA for 40 min. and photographed on the Foto
Eclipse™ and Foto Analyst® gel doc system (Fotodyne Inc., Hartland, WI).
Approximately 100 visualized bands from each DGGE gel were excised with sterile 1-ml pipet tips. TotalLab™ gel analysis software (Nonlinear Dynamics Ltd., Durham, NC) was used to identify distinct bands, determine band intensity (Rf value), calculate DNA quantity in each band by comparing Rf values in bands from the samples to bands of known DNA concentration, and cross compare operational taxonomic units (OTUs).

2.3.4 DNA Sequencing

One excised band representing each OTU was re-amplified as described above (PCR section) with GC-clamped primers. These PCR products were then subjected to DGGE to confirm that each excised band represented a single sequence. Once the bands were visually pure, they were re-amplified with non-GC-clamped primers, purified with the QIAquick® PCR Purification Kit (QIAGEN, Inc., Valencia, CA), and sent for commercial DNA sequencing (MWG Sequencing, High Point, NC) using the non-GC-clamped forward primer. The sequences were analyzed using the BLAST tool at the National Center for Biotechnology (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov) to identify the eukaryotic taxa present in our DNA samples. If DGGE bands returned the highest similarity with an uncultured environmental sample, the next highest similarity to a biologically relevant species is reported here. The DGGE band sequences were deposited onto GenBank under the accession numbers GU459011 through GU459054. Sequences were viewed in Chromas Lite, Version 2.01 (Technelysium Pty Ltd. 2005) and modified prior to submission such that bases labeled with “N” were replaced with more specific bases following the IUPAC-IUB codes.

2.3.5 Data Analysis and Statistics
The relative abundance of each OTU in each sample was calculated using the Rf values generated with the TotalLab™ gel analysis software. Some faint bands in the DGGE gels had Rf values of zero that were changed to 0.1 for analyses that included relative abundance. OTU richness and diversity were determined in each vernal pool to investigate how these metrics were related to urbanization, as there has been conflicting results from previous studies (see above). Richness was determined by counting the number of bands in each DGGE lane and the Shannon-Weaver diversity index was calculated as described by Nübel et al. (1999). Pearson correlations between percent urban area and richness and diversity were conducted in SigmaStat, Version 3.5 (Systat Software, Inc., 2006). Changes in OTU patterns, including the presence/absence of OTUs and relative band intensity, were identified by performing the ordination technique of nonmetric multidimensional scaling (NMS) with PC-ORD, Version 5.0 (Bruce McCune and MJM Software, 1999). Even though the data were proportions, the raw data were used for the analyses reported here because an arcsine, square root transformation did not change the distribution of the data nor did it considerably alter the results. NMS clusters were determined visually after examining all possible graphs (including two and three dimensional graphs) from the resulting three dimensional solutions. Multi-response permutation procedure (MRPP) and indicator species analysis were performed on the NMS clusters also in PC-ORD. MRPP was used to determine if the clusters differed statistically and indicator species analysis was used to identify specific OTUs that were possibly driving the clustering pattern. In order to determine if eukaryotic community patterns corresponded with the changing vernal pool environment, the general water quality parameters we measured were compared between the NMS clusters using t-tests.
(conducted in SigmaStat; Systat Software, Inc., 2006). If data were not normally distributed, a Mann-Whitney rank sum test was used in place of a parametric t-test. The water chemistry data were also compiled in a secondary matrix which was correlated with the NMS axes in PC-ORD. The resulting Pearson correlation coefficients allowed further investigation of the relationship between environmental parameters and microbial community structure. Richness, diversity, and NMS analyses were conducted using all microbial eukaryotic OTUs, as well as the subset of OTUs that were only non-photosynthetic protists (labeled protist), only autotrophic protists (labeled algae), or only fungi. Graphs were made in SigmaPlot, Version 10.0 (Systat Software, Inc., 2006). NMS clusters were labeled with E1 and E2 for the NMS ordination of all microbial eukaryotes. Likewise, the algal and fungal NMS clusters were labeled A1 and A2 and F1 and F2, respectively.

2.4 Results

DGGE revealed 47 OTUs in total from the 30 vernal pools. Successful DNA sequences were recovered from 44 of these OTUs, which were broadly organized into three major taxonomic groups: fungi, algae, and protists (Table 2.1). Of these 44 recovered sequences, 41% matched with fungal species, specifically the groups Ascomycota, Basidiomycota, and Chytridiomycota; 27% matched with protist species, specifically the groups Alveolata, Cercozoa, and Amoebozoa; and 23% matched with algal species, specifically the groups Bacillariophyceae, Chrysophyceae, Synurophyceae, and Xanthophyceae (Table 2.1). Any sequences of macroscopic eukaryotes (shown in Table 2.1) were removed from further analyses.
Vernal pools in more urbanized sub-watersheds tended to have higher eukaryotic richness and diversity (Figure 2.2 a and b). Eukaryotic diversity had a significant positive correlation with percent urban area (Pearson correlation coefficient = 0.523; p-value < 0.01). Similarly, there were positive trends in richness and diversity of protist, fungi, or algae OTUs with urbanization (Figure 2.2 c and d). Both fungal richness (Pearson correlation coefficient = 0.497; p-value < 0.01) and fungal diversity (Pearson correlation coefficient = 0.540; p-value < 0.01) were significantly positively correlated with percent urban area. No significant correlations were found between percent urban area and eukaryotic richness, algal richness or diversity, or protist richness or diversity.

Community composition in the vernal pools varied between the sub-watersheds. For all eukaryotic OTUs, the vernal pools formed two fairly distinct clusters in the NMS ordination (Figure 2.3a) that were shown to be significantly different by MRPP (p < 0.001). The vernal pools in these two clusters differed significantly in their surrounding urbanization (p = 0.036) and dissolved oxygen (p = 0.029), with cluster E1 pools having a lower percent urban area and higher dissolved oxygen concentration (Table 2.2). This suggests that the eukaryotic community changed in response to dissolved oxygen content, which can be lowered due to urban runoff. We also found a significantly lower C:N ratio in the leaves in cluster E1 pools (p = 0.037; Table 2.2). This differing nutrient content of the leaves could be due to a difference in canopy cover, as anecdotal data we collected using canopy pictures over the pools suggests that the main sources of leaf litter into cluster E1 pools were *Acer* trees, which are relatively easily decomposed, while the canopy trees over cluster E2 pools contained higher numbers of *Fraxinus*, *Quercus*, *Platanus*, and *Populus*. Several additional environmental variables were likely influential
in structuring the eukaryotic community, as the dimensions of the eukaryotic NMS were strongly correlated (i.e., Pearson correlation coefficients ≥ 0.296) with temperature, conductivity, the %C content of the leaves, and all land use categories (Table 2.3). Several OTUs were found to be significant indicators of the clusters with indicator species analysis. A diatom sequence (OTU 9) was a significant indicator of cluster E2 communities (Tables 2.1 and 2.2). Protist sequences OTU 11 and OTUs 8 and 26 were significant indicators of clusters E1 and E2, respectively (Table 2.2). The protist OTU that was a significant indicator for cluster E1 was a Cercozoa, while the protist indicators for cluster E2 were an Amoebozoa and a Cilophora (Table 2.1). Two fungal OTUs (numbers 34 and 38) were significant indicators for cluster E1, while cluster E2 had only one significant fungal indicator: OTU 35 (Table 2.2). All three of these OTUs matched with Ascomycota (Table 2.1).

When the individual groups were analyzed with NMS, both the fungi and the algae showed a cluster pattern (Figure 2.3b and c; a reliable NMS diagram was not generated with protist data). The fungal clustering was similar to the overall eukaryotic clustering, suggesting that the fungi may be strongly affecting the overall eukaryotic NMS. MRPP showed these clusters to also be significantly different (p < 0.001). The vernal pools in the fungal clusters differed in their conductivity (p = 0.001) and %C content of the leaves (p = 0.002), both of which were lower in cluster F1 (Table 2.2). The differing litter quality again corresponded with a predominantly maple canopy surrounding the cluster F1 pools. Further, the %C of leaves and water conductivity, along with water temperature and dissolved oxygen, were strongly correlated with fungal NMS dimensions (Table 2.3). There were several significant indicator OTUs for the
fungal clusters, all which matched with sequences in the group Ascomycota. Significant indicators for cluster F1 were OTUs 34 and 38, the former which matched with a root endophyte and the latter which matched with a mitosporic fungus (Tables 2.1 and 2.2). OTUs 35 and 45 (both matches with aquatic hyphomycetes) along with OTU 39 (a match with a plant pathogen) were significant indicators for fungal cluster F2 (Tables 2.1 and 2.2). The clustering of the algal NMS diagram showed six pools to differ significantly from the rest in their composition of algae (p < 0.001 with MRPP analysis). Only the conductivity was significantly different between the algal clusters (p = 0.022) and was lower in cluster A2 pools (Table 2.2). When the algal NMS dimensions were correlated with the secondary matrix, water temperature and dissolved oxygen, leaf %C and C:N, and all land use categories were found to be strongly correlated (Table 2.3). For the algal NMS, significant indicators for cluster A1 were OTUs 18 and 20 (both matches with golden algae of the Synurophyceae). For cluster A2, OTU 19 (a match with a golden algal species in the Chrysophyceae) and 23 (a match with a yellow-green algal species in the Xanthophyceae) were significant indicators (Table 2.2).

2.5 Discussion

The primary aim of this study was to determine whether the eukaryotic microbial community of vernal pools varied with urbanization at a landscape scale. We observed an increase in microbial diversity and, specifically, in fungal richness and diversity with increasing urbanization. The lack of eroded richness and diversity with human impact suggests that vernal pool microorganisms, which are tolerant to the widely varying environmental conditions of an ephemeral habitat (Carrino-Kyker and Swanson, 2007; Carrino-Kyker and Swanson, 2008), can also tolerate the environmental fluctuations
associated with urbanization. In some studies where macroscopic organisms have been surveyed, a positive relationship between human population density and species richness was found (Balmoford et al., 2001; reviewed in Arújo, 2007). It has been argued, at least for macroscopic organisms, that this positive relationship is due to the fact that humans, along with many other species, benefit by living in regions of high environmental heterogeneity (Luck, 2007; Hugo and van Rensburg, 2008). Urbanization may be increasing habitat heterogeneity in vernal pools, in both space and time, resulting in higher levels of diversity. Studies of stream bacterial (Cody et al., 2000) and ciliate (Dopheide et al., 2009) communities also found higher richness and diversity at sites more impacted by human activity. It is plausible that in urbanized watersheds increased nutrient runoff and changes in water chemistry following storm events create greater temporal heterogeneity in aquatic habitats, leading to increases in overall microbial diversity. Forest fragmentation and plant community changes in urban watersheds could also create spatial variability across a vernal pool in terms of light availability and water temperature, leading to greater within-pool heterogeneity and microbial diversity. For microorganisms, though, it may be that the effect of urbanization on richness and diversity is specific to different habitats or regions. Indeed, others have noted a decrease (e.g., Jumpponen and Jones, 2009) while a greater number of other studies have found no change in microbial richness and diversity with anthropogenic pollutants (e.g., Dorigo et al., 2002; Cousins et al., 2003; Duarte et al., 2004; Duong et al., 2007; Borin et al., 2009). Though metrics of richness and diversity can be useful, when applied to microbial communities, they are often insensitive of environmental changes (Morris and
Blackwood, 2007); thus the effects of urbanization reported here may be more strongly impacting microbial community composition.

It is more common that microbial communities change in their taxonomic composition with human impacts, which has been consistently documented in scientific literature (e.g., Cody et al., 2000; Dorigo et al., 2002; Cousins et al., 2003; Duarte et al., 2004; Duong et al., 2007; Borin et al., 2009; Dopheide et al., 2009). We also found that the community composition of eukaryotic microbes in vernal pools was related to the percent of each sub-watershed that was urban area. This observation is likely due to the fact that some species of protist, algae, and fungi are better adapted to the environmental conditions created by urbanization than others. That each eukaryotic cluster resulting from the NMS ordination had significant indicator OTUs associated with them lends support for the conclusion that some species are better adapted to the urban pools than others. This is further supported by the observations that the eukaryotic community structure was significantly affected by dissolved oxygen concentration, while the fungal and algal community structures were significantly affected by conductivity. These general water quality characteristics are known to be influenced by urbanization with more impacted aquatic habitats often experiencing lower dissolved oxygen concentration and higher conductivity due to polluted runoff from developed areas (Daniel et al., 2002; Miserendino et al., 2008).

The importance of dissolved oxygen and conductivity for eukaryotic microbes was also shown by the indicator species analysis. Of the seven indicator OTUs between the eukaryotic NMS groups, three were protists and three were fungi. In aquatic habitats, dissolved oxygen is of paramount importance for the distribution of protists, with most
species requiring high amounts of oxygen and others capable of tolerating low oxygen conditions (Wetzel, 2001). Aquatic fungi are also reliant on dissolved oxygen to carry out decomposition with different species thriving in high versus low oxygen conditions (Medeiros et al., 2009). In vernal pools, the conductivity likely includes measures of essential nutrients, namely phosphorus and nitrogen ions, which are often high in urban runoff (Carpenter et al., 1998). These nutrients are important determinants for the survival of different algal and fungal species. Phosphorus availability is particularly important for the distribution of golden-brown algae (Wetzel, 2001), which comprise three of the four significant OTUs for the algal NMS groups. Nutrient availability is also important for the decomposition activity of aquatic fungi (Gulis et al., 2006), and all significant OTUs for the fungal NMS groups were in the Ascomycota, a group that contains over 90% of all aquatic hyphomycetes (Webster, 1992). Vernal pool conductivity can also be related to increased salt content in runoff from road deicing agents (Karraker et al., 2008) and certain algal and fungal species may be more tolerant of these high salt levels.

It is worthwhile to note that urbanization is not the only factor known to affect dissolved oxygen or conductivity and other characteristics of the landscape may be important for vernal pools, as urbanization was no longer a significant factor in structuring the fungal and algal communities when they were examined alone. For example, conductivity can be affected by soil type that varies across the landscape (Wierenga et al., 1969). In the same 30 pools used in the current study, agricultural land use was found to be positively correlated with conductivity (Carrino-Kyker and Swanson, 2007). Though percent agriculture was not significantly different between any of the
NMS clusters in the current study, it was strongly correlated with both eukaryotic and algal NMS dimensions. Water temperature, which we found consistently correlated with NMS dimensions, affects the dissolved oxygen content of water (Wetzel, 2001) and can vary in shallow water bodies at multiple scales (e.g., spatially across a landscape as wetland area changes, Li et al., 2009, or temporally as air temperatures rise and fall, over the course of a day, Podrabsky et al., 1998, or season, Bonner et al., 1997). Regardless of the factors responsible for the change, our data indicate that the composition of eukaryotic microorganisms in vernal pools varied with urbanization at a landscape scale and such a change in microbial community composition may have important consequences for ecosystem function (Kinzig et al., 2001; Loreau et al., 2002; Morin and McGrady-Steed, 2004), which should be explored in future studies of these habitats.

Our results also suggest that local site-specific characteristics may be just as important as urbanization in structuring the eukaryotic community of vernal pools. We found the OTU composition of eukaryotic and fungal communities to be significantly influenced by litter quality and at least one dimension resulting from the eukaryotic, fungal, and algal NMS ordinations was strongly correlated with at least one aspect of litter quality. Similarly, Cousins et al. (2003) found that the structure of arbuscular mycorrhizal fungi was influenced by vegetation type just as much as by land use. Further, Ochimaru and Fukuda (2007) found that the richness of fungi involved in decomposition was higher in urban forests compared to rural forests and suggested that this was due to differential litter distribution, not the effects of land use. Contrary to our findings, Verb et al. (2001) saw little change in algal community structure with different vegetative litter substrates in a mesocosm study after 56 days of duration. Litter quality
may be more important for fungi than algae, as no significant differences in litter quality were seen between algal NMS clusters. Nevertheless, in landscape scale studies, the vegetation at a more local scale can not be ignored, as litter quality has been shown to affect both community structure as well as ecosystem function (Alarcón-Gutiérrez et al., 2009; Kotilainen et al., 2009; Ndaw et al. 2009). Our observation that the majority of OTU sequences were saprotrophic fungi, which are involved in the decomposition of allochthonous leaf litter into vernal pools, provides further support that litter quality and local environmental factors are important for vernal pool microbial community structure. Future studies should explore the influence of leaf litter composition and quality on vernal pool microbial communities, as they appear to play a role in structuring these communities that form the basis of the aquatic food web, but were not quantitatively measured in the current study.

2.6 Conclusions

Overall, we found that urbanization was associated with an increase in microbial richness and diversity and an altered microbial community composition in vernal pools. This suggests that there is a suite of eukaryotic microbial species which are able to tolerate the fluctuations in water chemistry associated with urbanization and are likely aided by their adaptations to the ephemeral environment of vernal pools. These changes in microbial community composition may also influence ecosystem function and future studies should investigate this. Urbanization was not the only factor found to affect microbial community composition. Litter quality and other local water chemistry changes may be just as important as larger landscape scale influences on vernal pool microbial communities. Therefore, when conducting landscape scale studies, local effects on the
microbial community can not be ignored, as they may be just as important an influence on community structure as landscape variability.
Table 2.1 The best matched biologically relevant DNA sequence from the GenBank database for each excised operational taxonomic unit (OTU).

<table>
<thead>
<tr>
<th>OTU#</th>
<th>Accession Number</th>
<th>Similarity</th>
<th>Best Database Match</th>
<th>Accession Number</th>
<th>Taxonomic Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GU459011</td>
<td>79%</td>
<td>Entamoeba chattoni</td>
<td>AF149912</td>
<td>Protist (Amoebozoa)</td>
</tr>
<tr>
<td>3</td>
<td>GU459012</td>
<td>94%</td>
<td>Entamoeba chattoni</td>
<td>AF149912</td>
<td>Protist (Amoebozoa)</td>
</tr>
<tr>
<td>5</td>
<td>GU459013</td>
<td>77%</td>
<td>Xiphecephalus triplogemmatus</td>
<td>FJ459763</td>
<td>Protist (Alveolata; Apicomplexa)</td>
</tr>
<tr>
<td>6</td>
<td>GU459014</td>
<td>80%</td>
<td>Cruoria pellita</td>
<td>AY437664</td>
<td>Rhodophyta</td>
</tr>
<tr>
<td>7</td>
<td>GU459015</td>
<td>95%</td>
<td>Loxophyllum rostratum</td>
<td>DQ190465</td>
<td>Protist (Alveolata; Ciliophora)</td>
</tr>
<tr>
<td>8</td>
<td>GU459016</td>
<td>79%</td>
<td>Amoeba leningradensis</td>
<td>AJ314605</td>
<td>Protist (Amoebozoa)</td>
</tr>
<tr>
<td>9</td>
<td>GU459017</td>
<td>98%</td>
<td>Navicula phylepta</td>
<td>FJ40068</td>
<td>Algae (Bacillariophyceae)</td>
</tr>
<tr>
<td>10</td>
<td>GU459018</td>
<td>98%</td>
<td>Synura uvella</td>
<td>U73222</td>
<td>Algae (Synurophyceae)</td>
</tr>
<tr>
<td>11</td>
<td>GU459019</td>
<td>96%</td>
<td>Heteromita globosa</td>
<td>U42447</td>
<td>Protist (Cercozoa)</td>
</tr>
<tr>
<td>12</td>
<td>GU459020</td>
<td>97%</td>
<td>Spongomonas minima</td>
<td>AF411280</td>
<td>Protist (Cercozoa)</td>
</tr>
<tr>
<td>13</td>
<td>GU459021</td>
<td>96%</td>
<td>Navicula brockmannii</td>
<td>AM502020</td>
<td>Algae (Bacillariophyceae)</td>
</tr>
<tr>
<td>14</td>
<td>GU459022</td>
<td>98%</td>
<td>Navicula gregaria</td>
<td>AM501974</td>
<td>Algae (Bacillariophyceae)</td>
</tr>
<tr>
<td>15</td>
<td>GU459023</td>
<td>94%</td>
<td>Chilodonella uncinata</td>
<td>AF300283</td>
<td>Protist (Alveolata; Ciliophora)</td>
</tr>
<tr>
<td>17</td>
<td>GU459024</td>
<td>95%</td>
<td>Mallomonas tonsurata</td>
<td>EF633325</td>
<td>Algae (Synurophyceae)</td>
</tr>
<tr>
<td>18</td>
<td>GU459025</td>
<td>93%</td>
<td>Spumella sp.</td>
<td>EF027354</td>
<td>Algae (Synurophyceae)</td>
</tr>
<tr>
<td>19</td>
<td>GU459026</td>
<td>96%</td>
<td>Hydrurus foetidus</td>
<td>FM955256</td>
<td>Algae (Chrysophyceae)</td>
</tr>
<tr>
<td>20</td>
<td>GU459027</td>
<td>95%</td>
<td>Spumella sp.</td>
<td>AJ236858</td>
<td>Algae (Synurophyceae)</td>
</tr>
<tr>
<td>21</td>
<td>GU459028</td>
<td>97%</td>
<td>Phagocata sibirica</td>
<td>Z99948</td>
<td>Platyhelminthes</td>
</tr>
<tr>
<td>22</td>
<td>GU459029</td>
<td>94%</td>
<td>Chilodonella uncinata</td>
<td>AF300283</td>
<td>Protist (Alveolata; Ciliophora)</td>
</tr>
<tr>
<td>23</td>
<td>GU459030</td>
<td>98%</td>
<td>Tribonema utriculosum</td>
<td>AM490826</td>
<td>Algae (Xanthophyceae)</td>
</tr>
<tr>
<td>24</td>
<td>GU459031</td>
<td>98%</td>
<td>Gomphonema angustatum</td>
<td>AM502005</td>
<td>Algae (Bacillariophyceae)</td>
</tr>
<tr>
<td>25</td>
<td>GU459032</td>
<td>91%</td>
<td>Lecithysm sp.</td>
<td>AJ514867</td>
<td>Protist (Cercozoa)</td>
</tr>
<tr>
<td>26</td>
<td>GU459033</td>
<td>98%</td>
<td>Hypotrichida sp.</td>
<td>AF508777</td>
<td>Protist (Alveolata; Ciliophora)</td>
</tr>
<tr>
<td>27</td>
<td>GU459034</td>
<td>95%</td>
<td>Oxytricha sp.</td>
<td>AF508769</td>
<td>Protist (Alveolata; Ciliophora)</td>
</tr>
<tr>
<td>28</td>
<td>GU459035</td>
<td>95%</td>
<td>Chalara cylindrosperma</td>
<td>AF222507</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>29</td>
<td>GU459036</td>
<td>97%</td>
<td>Phagocata sibirica</td>
<td>Z99948</td>
<td>Platyhelminthes</td>
</tr>
<tr>
<td>30</td>
<td>GU459037</td>
<td>98%</td>
<td>Chalara constricta</td>
<td>AF222506</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>31</td>
<td>GU459038</td>
<td>96%</td>
<td>Podosphaera tridactyla</td>
<td>AB022392</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>32</td>
<td>GU459039</td>
<td>93%</td>
<td>Blytomyces helicus</td>
<td>DQ536491</td>
<td>Fungi (Chytridiomycota)</td>
</tr>
<tr>
<td>33</td>
<td>GU459040</td>
<td>88%</td>
<td>Chalara sessilis</td>
<td>AF222514</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>34</td>
<td>GU459041</td>
<td>98%</td>
<td>Phialocephala sp.</td>
<td>EU155146</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>35</td>
<td>GU459042</td>
<td>98%</td>
<td>Tetracladium sp.</td>
<td>FJ53447</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>36</td>
<td>GU459043</td>
<td>96%</td>
<td>Microdochum nivale</td>
<td>AF548077</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>37</td>
<td>GU459044</td>
<td>96%</td>
<td>Buellia georgeri</td>
<td>AJ421681</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>38</td>
<td>GU459045</td>
<td>98%</td>
<td>Madurella sp.</td>
<td>EU815932</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>39</td>
<td>GU459046</td>
<td>88%</td>
<td>Pycnobaena lycopersici</td>
<td>DQ898289</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>40</td>
<td>GU459047</td>
<td>96%</td>
<td>Ophiocordyceps sinensis</td>
<td>EF555097</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>41</td>
<td>GU459048</td>
<td>98%</td>
<td>Thanatephorus cucumeris</td>
<td>DQ917659</td>
<td>Fungi (Basidiomycota)</td>
</tr>
<tr>
<td>42</td>
<td>GU459049</td>
<td>98%</td>
<td>Carallia brachiata</td>
<td>AM235548</td>
<td>Viridiplantae; Magnoliophyta</td>
</tr>
</tbody>
</table>
Table 2.1 (con’td)

<table>
<thead>
<tr>
<th>OTU#</th>
<th>Accession Number</th>
<th>Similarity</th>
<th>Best Database Match</th>
<th>Accession Number</th>
<th>Taxonomic Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>GU459050</td>
<td>98%</td>
<td>Aureobasidium pullulans</td>
<td>FJ023536</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>44</td>
<td>GU459051</td>
<td>99%</td>
<td>Boletus rubropunctus</td>
<td>FJ480426</td>
<td>Fungi (Basidiomycota)</td>
</tr>
<tr>
<td>45</td>
<td>GU459052</td>
<td>95%</td>
<td>Thecotheus holmskjoldii</td>
<td>AF010589</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>46</td>
<td>GU459053</td>
<td>98%</td>
<td>Myxotrichum deflexum</td>
<td>AY541481</td>
<td>Fungi (Ascomycota)</td>
</tr>
<tr>
<td>47</td>
<td>GU459054</td>
<td>99%</td>
<td>Mrakia frigida</td>
<td>DQ831017</td>
<td>Fungi (Basidiomycota)</td>
</tr>
</tbody>
</table>

*a* Considered a macroscopic organism and removed from statistical analyses.

*b* Any OTU that had an 80% or lower match to a sequence in the NCBI database were considered to have an unknown taxonomic identity.
### Table 2.2: Descriptive statistics for vernal pool environmental parameters and significant indicator species of each nonmetric multidimensional scaling (NMS) cluster.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Eukaryote NMS</th>
<th>Fungi NMS</th>
<th>Algae NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cluster E1 (n = 8)</td>
<td>Cluster E2 (n = 22)</td>
<td>p-value&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH</td>
<td>7.54 ± 0.38</td>
<td>7.62 ± 0.12</td>
<td>0.944</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg l&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>11.72 ± 1.08</td>
<td>8.08 ± 0.87</td>
<td>0.029*</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>7.26 ± 0.54</td>
<td>6.07 ± 0.85</td>
<td>0.467</td>
</tr>
<tr>
<td>Conductivity (µmho cm&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>134.13 ± 35.59</td>
<td>288.68 ± 46.46</td>
<td>0.064</td>
</tr>
<tr>
<td>Depth (cm)</td>
<td>7.41 ± 1.29</td>
<td>8.07 ± 0.82</td>
<td>0.677</td>
</tr>
<tr>
<td>% N</td>
<td>1.48 ± 0.13</td>
<td>1.31 ± 0.068</td>
<td>0.218</td>
</tr>
<tr>
<td>% C</td>
<td>33.15 ± 1.43</td>
<td>37.19 ± 8.84</td>
<td>0.227</td>
</tr>
<tr>
<td>C:N</td>
<td>23.11 ± 1.36</td>
<td>29.41 ± 1.65</td>
<td>0.037*</td>
</tr>
<tr>
<td>% Urban Area</td>
<td>22.12 ± 6.09</td>
<td>45.07 ± 5.58</td>
<td>0.036*</td>
</tr>
<tr>
<td>% Agriculture</td>
<td>16.84 ± 2.58</td>
<td>12.40 ± 1.27</td>
<td>0.094</td>
</tr>
<tr>
<td>% Natural Area</td>
<td>61.04 ± 4.90</td>
<td>42.38 ± 5.12</td>
<td>0.051</td>
</tr>
<tr>
<td>Indicator Species&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--</td>
<td>OTU 8</td>
<td>0.008*</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>OTU 9</td>
<td>0.035*</td>
</tr>
<tr>
<td>OTU 11</td>
<td>--</td>
<td>--</td>
<td>0.0006*</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>OTU 26</td>
<td>0.028*</td>
</tr>
<tr>
<td>OTU 34</td>
<td>--</td>
<td>--</td>
<td>0.0008*</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>OTU 35</td>
<td>0.0002*</td>
</tr>
<tr>
<td>OTU 38</td>
<td>--</td>
<td>--</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values for the environmental parameters are mean ± standard error.

<sup>b</sup>p-values for the environmental parameters were determined with t-tests, while the p-values for the indicator species were determined with indicator species analysis.

<sup>c</sup>Only significant indicator species are shown. The identity of each of these operational taxonomic units is listed in Table 2.1.

*Indicates a significant p-value.
Table 2.3 Pearson correlation coefficients showing the relationship between vernal pool characteristics (e.g., water chemistry, substrate litter quality, and sub-watershed land use) and nonmetric multidimensional scaling (NMS) dimensions (labeled as “Dim”). NMS was performed for the entire eukaryotic microbial community (labeled “Eukaryote”) as well as the portion of the community identified as fungi (labeled “Fungi”) or autotrophic protists (labeled “Algae”). For the purposes of this study, strong correlations were identified as any Pearson correlation coefficient ≥ 0.296 (Zar, 1999).

<table>
<thead>
<tr>
<th></th>
<th>Eukaryote Dim 1</th>
<th>Eukaryote Dim 2</th>
<th>Eukaryote Dim 3</th>
<th>Fungi Dim 1</th>
<th>Fungi Dim 2</th>
<th>Fungi Dim 3</th>
<th>Algae Dim 1</th>
<th>Algae Dim 2</th>
<th>Algae Dim 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>-0.094</td>
<td>0.100</td>
<td>0.132</td>
<td>-0.247</td>
<td>-0.075</td>
<td>-0.115</td>
<td>0.045</td>
<td>-0.035</td>
<td>-0.153</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg l(^{-1}))</td>
<td>0.066</td>
<td>-0.262</td>
<td>0.226</td>
<td>0.348</td>
<td>0.045</td>
<td>-0.055</td>
<td>-0.089</td>
<td>-0.071</td>
<td>0.324</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>-0.349</td>
<td>-0.334</td>
<td>-0.048</td>
<td>0.064</td>
<td>0.158</td>
<td>-0.492</td>
<td>0.379</td>
<td>-0.259</td>
<td>-0.259</td>
</tr>
<tr>
<td>Conductivity (µmho cm(^{-1}))</td>
<td>-0.332</td>
<td>0.081</td>
<td>-0.404</td>
<td>-0.201</td>
<td>0.247</td>
<td>-0.412</td>
<td>0.244</td>
<td>0.220</td>
<td>-0.181</td>
</tr>
<tr>
<td>Depth (cm)</td>
<td>0.029</td>
<td>-0.291</td>
<td>-0.201</td>
<td>-0.100</td>
<td>0.166</td>
<td>0.049</td>
<td>0.170</td>
<td>0.187</td>
<td>-0.205</td>
</tr>
<tr>
<td>% N</td>
<td>-0.120</td>
<td>-0.227</td>
<td>-0.066</td>
<td>0.022</td>
<td>0.155</td>
<td>-0.180</td>
<td>0.111</td>
<td>-0.115</td>
<td>0.135</td>
</tr>
<tr>
<td>% C</td>
<td>-0.246</td>
<td>-0.016</td>
<td>-0.302</td>
<td>-0.329</td>
<td>0.277</td>
<td>-0.213</td>
<td>0.096</td>
<td>-0.244</td>
<td>-0.329</td>
</tr>
<tr>
<td>C:N</td>
<td>-0.087</td>
<td>0.211</td>
<td>-0.165</td>
<td>-0.272</td>
<td>0.047</td>
<td>-0.004</td>
<td>-0.136</td>
<td>-0.068</td>
<td>-0.310</td>
</tr>
<tr>
<td>% Urban Area</td>
<td>-0.237</td>
<td>0.469</td>
<td>0.098</td>
<td>-0.244</td>
<td>-0.145</td>
<td>-0.078</td>
<td>-0.335</td>
<td>0.109</td>
<td>0.397</td>
</tr>
<tr>
<td>% Agricultural Area</td>
<td>0.184</td>
<td>-0.431</td>
<td>-0.224</td>
<td>0.283</td>
<td>0.232</td>
<td>0.054</td>
<td>0.333</td>
<td>0.268</td>
<td>-0.234</td>
</tr>
<tr>
<td>% Natural Area</td>
<td>0.210</td>
<td>-0.404</td>
<td>-0.047</td>
<td>0.193</td>
<td>0.098</td>
<td>0.067</td>
<td>0.286</td>
<td>-0.196</td>
<td>-0.377</td>
</tr>
</tbody>
</table>
Figure 2.1 GIS characterized land use of 10 sub-watersheds of the Cuyahoga River Basin, OH, USA. The percent urbanization of each sub-watershed was defined as the sum of percent urban, percent suburban, and percent barren area (the lowest three categories on the bars, represented by solid black or solid gray colors). Sub-watershed numbers were assigned in the order of increasing percent natural area, which included percent wooded, percent shrub/scrub, and percent streams/open water. (Figure from Carrino-Kyker and Swanson, 2007.)
Figure 2.2 Average richness and Shannon Weaver diversity (± standard error) of the entire eukaryotic community (a and b) as well as the subset of operational taxonomic units that were fungi, algae, or protists (c and d) found in 30 vernal pools across a gradient of urbanization. The vernal pools were located in ten sub-watersheds with differing degrees of percent urban area (n=3 pools per sub-watershed). Richness and diversity were determined with denaturing gradient gel electrophoresis.
Figure 2.3 Three dimensional nonmetric multidimensional scaling (NMS) ordination diagrams showing the similarity between the vernal pool a) eukaryotic community, b) fungal community, and c) algal community. The stress values for each NMS were 13.70% for the eukaryotic ordination (a), 13.78% for the fungal ordination (b), and 11.34% for the algal ordination (c). Each pool is represented by a number indicating the sub-watershed within which that pool was located. On each graph are two clusters which are identified by circles and labeled with an E, F, or A for the eukaryotic, fungal, and algal NMS ordinations, respectively. The clusters were visually determined and found to be significantly different in their community structure with multi-response permutation procedure.
3.1 Summary

How ecosystem processes respond to environmental perturbation may depend on the level of functional redundancy among species within an ecosystem. However, species responses may depend on the nature of the perturbation and the type of community being examined. Some perturbations may affect entire communities, while others may affect only a subset of the community. In a vernal pool microcosm study, we found that microbial community structure and function responded differently to two environmental changes: pH manipulation and nitrate (NO\textsubscript{3}\textsuperscript{-}) addition. The pH treatment included manipulation of the microcosms to values of 5, 6, 7, or 8, while the NO\textsubscript{3}\textsuperscript{-} addition was accompanied by two control treatments (an ammonium (NH\textsubscript{4}\textsuperscript{+}) and distilled water additions) in a 4x3 design. The community structure of bacteria and fungi was altered by pH, which corresponded with significant differences in microbial respiration. This suggests that environmental changes which influence the microbial communities broadly, such as pH, can affect both microbial community structure and function. NO\textsubscript{3}\textsuperscript{-} addition had no affect on bacterial, fungal, or denitrifier community structure or on the population size or relative abundance of denitrifiers. However, elevated denitrification rates were only detected in microcosms with NO\textsubscript{3}\textsuperscript{-} addition. This indicates that changes in the availability of terminal electron acceptors may not affect community structure, population size, or abundance, even of functional groups. Rather, gene expression may
up-regulate, resulting in changes in ecosystem function without altered microbial community structure. No significant interaction between pH manipulation and $\text{NO}_3^-$ addition on denitrification rate or denitrifier community structure was observed, even though it was expected, suggesting that there may not be a pH optimum for denitrification. Finally, microcosms in the $\text{NH}_4^+$ addition treatment tended to have higher community respiration than those which received $\text{NO}_3^-$ or distilled water and had an increase in denitrifier population size similar to that observed in the $\text{NO}_3^-$ treatment. This indicates that $\text{NO}_3^-$ addition not only influences microbial communities due to its use as a terminal electron acceptor for denitrification, but can also broadly affect these organisms due to the removal of nutrient N limitation. Overall, our results suggest that community stability and effects on ecosystem function are highly context-dependent, driven by the nature of environmental perturbation and the physiological plasticity of the communities affected.

3.2 Introduction

Given the predicted effects of global environmental changes on the ecology of organisms and ecosystem processes, it is increasingly important to characterize the relationship between community structure and ecosystem function (Tilman et al., 2001; Zak et al., 2006). Current studies in ecology have attempted to make mechanistic linkages between environmental conditions and species distribution, and to understand how underlying ecosystem processes are affected by species composition (reviewed in Kinzig et al., 2001 and Loreau et al., 2002). It has been suggested that systems which have multiple species exhibiting similar ecological functions (a natural condition known as functional redundancy; Walker, 1992; Lawton and Brown, 1993) are more resistant to
environmental change because the functional properties of the system are distributed among a larger number of taxa, many of which perform optimally under different environmental conditions (e.g., Naeem and Li, 1997; Tilman et al., 1997). Thus, the presence of many species with redundant functional attributes in natural systems could be necessary to ensure ecosystem resilience to environmental perturbations (Walker, 1995), such as those resulting from global environmental change (e.g., climate change, acid deposition, nitrogen deposition, urban sprawl). A great deal of global change research (Tilman et al., 2001; Morgan et al., 2007; Buisson et al., 2008; Ibanez et al., 2008) and functional redundancy research (Tilman and Downing, 1994; Tilman et al., 1997; Micheli and Halpern, 2005; O’Connor and Crowe, 2005) has focused on plant or animal communities. Much less attention has centered on microbial communities, even though these communities often underlie many ecosystem processes.

Microbial communities exert a strong control on soil carbon and nutrient cycling. Therefore, changes in microbial community abundance, composition, or activity in response to environmental change could have dramatic impacts on ecosystem processes (Prosser, 2002). For microbial communities, however, the relationship between community structure and function may be context-dependent with environmental modulators (i.e. abiotic conditions) and resources differentially affecting microbial communities and the ecosystem processes they perform (Balser et al., 2001; see Chapter 1 for a discussion of this conceptual model). The relationship between microbial community structure and ecosystem function, therefore, may be dependent on the environmental perturbation and community being examined.
In the current study, we examined the paradigm of how fluctuating modulators and resources influence microbial communities in a laboratory-based microcosm experiment. Vernal pools were replicated in the laboratory and subjected to pH manipulation and one of three nitrogen addition treatments (Table 3.1). Vernal pools were chosen because they are spatially and temporally dynamic (reviewed in Colburn, 2004), which makes them an ideal model system for studying environmental change. Environmental modulators, such as pH, water temperature, dissolved oxygen, and depth, fluctuate widely and rapidly over the hydroperiod of these shallow, temporary ponds (Carrino-Kyker and Swanson, 2007; Carrino-Kyker and Swanson, 2008), which contributes to a spatially and temporally dynamic microbial community (Carrino-Kyker and Swanson, 2008; Chapter 2). For example, pH was found to vary by 3-4 pH units throughout the spring season in thirty-five vernal pools in Northeastern Ohio (Carrino-Kyker and Swanson, 2007; Carrino-Kyker and Swanson, 2008). Previous data demonstrated that pH is correlated with the bacterial and microbial eukaryotic composition of vernal pools (Carrino-Kyker and Swanson, 2008) and is an important predictor of bacterial composition in soils sampled from six different ecosystems across North and South America (Fierer and Jackson, 2005). In addition, vernal pools are recharged by runoff and groundwater (Colburn, 2004) which can contain high levels of nitrate (NO$_3^-$) pollution (Moldan and Černý, 1994). In low oxygen environments, such as vernal pools, NO$_3^-$ enrichment often leads to an increase in denitrification, the reduction of NO$_3^-$ and nitrite (NO$_2^-$) to nitrous oxide (N$_2$O) and nitrogen gas (N$_2$) in the pathway: NO$_3^-$ → NO$_2^-$ → NO → N$_2$O → N$_2$. Thus, NO$_3^-$ enrichment, along with acid deposition,
has a high potential to influence the ecology and biogeochemistry of vernal pools at both the pool and landscape-scale.

Based on the Balser et al. (2001) model, we predicted that pH (i.e., modulator) would broadly affect the microbial community structure (i.e., influence all bacteria and all fungi), but no effects of pH manipulation would be realized when only the functional group (i.e., denitrifying organisms) or the function performed (i.e., denitrification) were analyzed. However, the addition of NO$_3^-$ (i.e., resource) was predicted to alter the community structure of denitrifying organisms (which use NO$_3^-$ as a terminal electron acceptor), increase their population size and relative abundance in the community, and, ultimately, amplify the denitrification rate, while the broad communities would remain constant. Besides serving as a terminal electron acceptor, NO$_3^-$ could also remove N limitation in our microcosms. Though we did not expect the microbial communities to be altered by nutrient addition because the amount of NO$_3^-$ added was similar to natural levels, we included an ammonium (NH$_4^+$) addition treatment to determine if any changes observed in the +NO$_3^-$ treatment were due to the removal of N limitation and not to the elevated presence of a terminal electron acceptor. On day 30 and day 31 of the study, we isolated communities of general bacteria, fungi, and denitrifiers with polymerase chain reaction (PCR), profiled microbial richness, diversity, and community structure using terminal restriction fragment length polymorphism (TRFLP), and measured denitrifier population size and relative abundance with quantitative PCR (QPCR). The denitrifier community in our microcosms was analyzed by amplifying *nosZ*, the gene for N$_2$O reductase, because this gene region was recently shown to exhibit high denitrifier diversity with TRFLP (Rösch et al., 2002; Rich et al., 2003).
3.3 Materials and Methods

3.3.1 Microcosm Set Up

Soil from four vernal pools, located at Squire Valleeve and Valley Ridge Farms, Hunting Valley, OH (41°29'53"N, 81°25'27"W; elevation, 320 m) was randomly sampled on 28 November, 2007. All pools were flooded with a low amount of standing water. We collected the top 2-5 cm layer of soil (where the majority of biota resting stages are found; Angeler et al., 2004) with a clean shovel and transported it to the laboratory in plastic bins that were impermeable to light. Once in the laboratory (approximately 3 hours later), the soil was transferred to flat plastic trays, mixed to eliminate bias by location, covered with shade cloth, and left to dry at room temperature. Once dry, the soil was sieved with a 2-mm sieve and stored at 4°C until use in the microcosms.

Freshly-fallen Fagus grandifolia Ehrh. leaves were also sampled on 28 November, 2007 from a mature Beech-Maple forest located at The Holden Arboretum, Kirtland, OH (41°36’40”N, 81°17’30”W; elevation, 340 m). Only F. grandifolia leaves were used in order to control for variability that could be introduced from using leaves of varying litter quality. The leaves were collected from the forest floor by hand, placed in plastic bins, mixed to eliminate bias by location, covered with shade cloth, and left to dry at room temperature. Once dry, 1.5-cm cores were punched from the leaves using a cork corer and the discs were stored at 4°C until use in the microcosms. All sampling equipment was sterilized before use.

3.3.2 Microcosm Treatments

In February of 2008, 72 microcosms were constructed in 500-mL Mason jars. Each microcosm received an equal weight (50 g) of sieved vernal pool soil and fine-grained
sterile sand (QUIKRETE® Premium Sand (No. 1113), Atlanta Georgia) that were mixed prior to their addition, 800 mg of leaf punches, and 150 mL of autoclaved distilled water. The sand was added to allow movement of gases and other materials through the medium that may not have been maintained if only the dried, sieved soil were included in the microcosms. An additional 200 mg of leaf discs were added in the form of 4 cm x 3 cm nylon mesh litter bags (600 µm mesh size). The microcosms were assigned to one of twelve treatments, with six replicates per treatment, which included four pH treatments and three nitrogen treatments in a 4 x 3 design (Table 3.1). The four pH treatments represented values detected in studies from Northeastern Ohio vernal pools (Carrino-Kyker and Swanson, 2007; Carrino-Kyker and Swanson, 2008) and were manipulated by low-volume addition of 5M sodium hydroxide (NaOH) to the microcosms. Based on amounts determined by titrations in a pilot study, the pH 6, 7, and 8 microcosms initially received 100 µL, 350 µL, and 500 µL, respectively, of 5M NaOH. Throughout the remainder of the study, 5M hydrochloric acid (as in Chu et al., 2007) and 5M NaOH were used to maintain the pH of each microcosm, which was monitored bi-weekly, at least. Some of the pH values recorded throughout the study are reported in Table 3.2. While HCl and NaOH may artificially raise salinity, measurements of sodium ions on day 113 and chloride ions on day 114 indicated that their levels in the microcosms were within the range of fresh water salinity (McArthur, 2006) and we expect the effects on organisms in the microcosms to be minimal compared to our experimental treatments. On day 29 of the experiment, the nitrogen treatments were implemented, such that each microcosm received a 20 mL solution of either 0.71 mmol NO₃⁻ in the form of KNO₃, 0.71 mmol NH₄⁺ in the form of (NH₄)₂SO₄, or sterile distilled water. The NO₃⁻ added
was similar to levels measured in vernal pools in Pennsylvania (Laposata and Dunson, 1998). The NH₄⁺ addition was used as a comparison to determine whether any treatment effects were due to low nitrogen (nutrient) availability, and the distilled water treatment was a control. The microcosms were randomly placed in an incubator at 16 ± 1°C with a 12 hr. light/12 hr. dark cycle, which is similar to the temperature and natural day/night cycle in Northeastern Ohio in the spring, and rotated within the incubator to limit bias of location. The jars were covered with parafilm that included vents to keep them open to air exchange. The pH of each microcosm was monitored throughout the study with a handheld meter (Oakton® Instruments, Vernon Hills, IL).

3.3.3 Ecosystem Functions and Water Chemistry
Microbial community respiration was determined on days 1, 4, 8, 15, 22, 29, 30, 31, 94, and 122 by measuring CO₂ efflux from each microcosm with a portable open-flow infrared gas analyzer (IRGA; LI-6400; LiCOR® Instruments, Lincoln, NE). Respiration was determined by scrubbing CO₂ from the microcosm headspace and measuring the change in headspace CO₂ concentration over time. We used the average of two separate scrub/flux cycles and a rate was calculated using the following formula:

\[
\text{Community Respiration} = \frac{(dc'/dt \times \text{system volume})}{\text{soil carbon}}
\]

where \(dc'/dt\) was the rate of change of CO₂ over 10 seconds determined with the IRGA and corrected for water content, system volume included the volume of microcosm headspace and IRGA tubing, and soil carbon was the mass of carbon per gram of dried soil added to the microcosms. The final respiration value was converted to mg CO₂-C * g soil C⁻¹ * day⁻¹. Statistics on respiration rate were not conducted until day 8 to allow for carbonate equilibrium (Wetzel, 2001).
Water color was determined spectrophotometrically in a subset of microcosms on day 28 following Rand et al. (1976). Decomposition of the leaf litter was determined by obtaining a dry weight of the litter bags at the end of the experiment (day 127). The litter bags were air dried for nine days prior to weighing.

Potential denitrification rates were determined with a modified acetylene-block technique (Groffman et al., 1999). On day 29, when the nitrogen treatments were administered, the microcosms were closed with airtight lids that were fitted with rubber septa and cycled five times between vacuum and purging with N\textsubscript{2} to induce anoxia. To each jar, acetylene was added to the soil (30 mL) and to the head space (40 mL). Between day 29 and day 31 of the experiment, 20 mL of headspace was sampled from each microcosm at 1 hour, 12 hours, 16 hours, 24 hours, 36 hours, and 40 hours following the nitrogen additions, and stored in scintillation vials fitted with airtight rubber stoppers (GeoMicrobial Technologies, Ochealata, OK). The N\textsubscript{2}O concentration of each headspace sample was measured with a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD) using an electron capture detector. Based on our standards, the lowest detection limit was 1.04 ppm N\textsubscript{2}O. Denitrification rate was calculated following Groffman et al. (1999) using the formula:

$$DR = \frac{[(C_2 \times H) - (C_1 \times H)]}{(D \times T)}$$

where

- DR = denitrification rate expressed as $\mu$g N * kg\textsuperscript{-1} * d\textsuperscript{-1}
- $C_1$ = N\textsubscript{2}O concentration at the first sampling time, expressed as $\mu$g N\textsubscript{2}O-N/L
- $C_2$ = N\textsubscript{2}O concentration at the second sampling time, expressed as $\mu$g N\textsubscript{2}O-N/L
- H = headspace volumes
D = dry soil mass

T = time between sampling points

The denitrification rates on day 30 and day 31 were calculated using three different sets of numbers for \( C_1 \) and \( C_2 \). For day 30, these sets of numbers were the 1 hour / 12 hour, 1 hour / 16 hour and 12 hour / 16 hour \( \text{N}_2\text{O} \) concentrations for day 30 and the 24 hour / 36 hour, 24 hour / 40 hour, and 36 hour / 40 hour \( \text{N}_2\text{O} \) concentrations for day 31.

3.3.4 DNA Extraction and PCR

On days 30 and 31, the jars were opened up to randomly sample one leaf disc and soil from two locations for microbial community analyses. Samples from two microcosms in the same treatment were pooled prior to DNA extraction, such that half of a leaf disc and \( \sim 125 \) mg of soil from each microcosm were used for the molecular work. (Therefore, \( n=3 \) soil and leaf samples per treatment group for the microbial analyses.) DNA was extracted separately from soil and leaf samples using the PowerSoil™ DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s protocol. A Precellys 24 Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) was used for cell lysis.

Template DNA from soil samples was diluted such that each PCR reaction received approximately 10 ng. DNA from the leaf samples was added undiluted so that each reaction received somewhere in the range of an undetected amount and 10 ng of template. The general bacteria PCR used the domain primers 338f (labeled with HEX) and 926r (labeled with 6FAM) to target the 16S rRNA gene (Burke et al., 2006). Each 50-µL PCR reaction contained 0.2 µM of each primer, 2.0 mM MgCl₂, 0.8 mM dNTP, 1X buffer, and 1.0 U Flexi Go Taq DNA polymerase (Promega Corporation, Madison,
The temperature cycling conditions were as described by Burke et al. (2006). The fungal PCR amplified the ITS region using the primer set 58A2F (labeled with HEX) and ITS4 (Burke et al., 2005). Each reaction contained 0.8mM dNTP mix, 0.2 µM of each primer, 1X buffer containing 2 mM MgCl₂, and 1.0 U of FastStart Taq polymerase (Roche Diagnostics, Mannheim, Germany) in a 50-µL reaction. The temperature cycling conditions for the fungal PCR were as described by Burke et al. (2005) except the extension step was 90 s. The nosZ gene was targeted for the denitrifying community PCR using the primers nosZFB and nosZRb (Rösch and Bothe, 2005). Each PCR reaction contained 0.8mM dNTP mix, 0.2 µM of each labeled primer (6FAM for nosZFB and Hex for nosZRb), 1X buffer containing 2 mM MgCl₂, and 1.0 U of FastStart Taq polymerase (Roche Diagnostics, Mannheim, Germany) in a 50-µL reaction. The temperature cycling for the nosZ PCR were as follows: 94°C initial denaturing step for 5 min. followed by 40 cycles of 94°C denaturing for 30 s, 1 min. of annealing at temperatures ranging between 65°C and 50°C, and a 72°C extension for 1 min., and a final extension at 72°C for 10 min. The annealing step was 65°C for one cycle, 62°C for the following two cycles, 59°C for the subsequent three cycles, 56°C for the next four cycles, 53°C for the following five cycles, and 50°C for the remaining 25 cycles (following Rösch et al., 2002). All PCR reactions were carried out on a PTC 100 Thermal Cycler (MJ Research, Boston, MA).

3.3.5 TRFLP

In order to examine the diversity and structure of the bacterial, fungal, and denitrifying communities, PCR products were digested with restriction enzymes for ~20 hours and subjected to TRFLP. The endonucleases MspI and HaeIII were used for bacterial TRFLP.
and AluI and HaeIII were used for fungal TRFLP. The denitrifier TRFLP was conducted using the restriction endonucleases MboI and TaqI because these enzymes were found to generate a high diversity of potential TRFs on the nosZ gene fragment (Rösch and Bothe, 2005). TRFLP profiles were generated at the Life Sciences Core Laboratories Center (Cornell University) and analyzed using the GS600 LIZ size standard and Peak Scanner™ Software (version 1.0, Applied Biosystems, 2006). For our analyses, only peaks that accounted for greater than 1% of the relative peak area were included (i.e., major TRFs).

3.3.6 QPCR

QPCR was conducted to determine copy number and relative abundance of the nosZ gene on a MiniOpticon™ System (Bio-Rad Laboratories, Inc., Hercules, CA). Only soil samples were used for QPCR, as the nosZ copy number in leaf samples were below the lowest level of detection. Each 25µl reaction contained 0.2 µM of each primer, 0.5 mg/mL BSA, 2µl template DNA, and 12.5µl of FastStart Universal SYBR Green Master (ROX), which contained FastStart DNA Taq polymerase, nucleotide mix, reaction buffer, SYBR Green 1 dye, and ROX reference dye (Roche Diagnostics, Mannheim, Germany). The temperature cycling included a 10 min. initial denaturation step at 95°C, 35 cycles of 95°C for 30 s for denaturing, 53°C for 1 min. for annealing, 72°C for 1 min. for extension, and 78°C (above the melting point of primer dimmers) for 45 s for primer degradation, and a final extension of 10 min. at 72°C. Fluorescence was measured at the end of each primer degradation step. Reactions were confirmed to be pure with agarose gel electrophoresis. The threshold cycle (Ct) was calculated for each run as the standard deviation of the fluorescence in the blank reactions (i.e., no template) for cycles 3
through 15. The copy number for each microcosm sample was determined in Opticon Monitor, Version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA) by comparing to a standard curve. The standard curve included $10^3$, $10^4$, $10^5$, and $10^6$ copies of the nosZ fragment inserted into pGEM®-T Easy Vectors (Promega Corporation, Madison, WI) that were linearized with the restriction enzyme PstI (New England BioLabs, Inc., Ipswich, MA) prior to construction of the standards. Relative abundance of nosZ–containing organisms was determined by dividing the copy number by the amount of DNA (in ng) extracted from the soil sample. Both denitrifier population size and relative abundance were estimated by using nosZ copy number per gram of soil or per ng of extracted DNA/g of soil, respectively, in the following formula:

$$C_{\text{dry}} = C_{\text{wet}} \times \left(\frac{100}{(100 - \%\text{moisture})}\right)$$

where

$$C_{\text{dry}} = \text{nosZ copy number} \times (\text{g dry soil})^{-1}$$

or $\text{nosZ copy number} \times (\text{ng DNA extracted})^{-1} \times (\text{g dry soil})^{-1}$

$$C_{\text{wet}} = \text{nosZ copy number} \times (\text{g wet soil})^{-1}$$

or $\text{nosZ copy number} \times (\text{ng DNA extracted})^{-1} \times (\text{g wet soil})^{-1}$

$$\% \text{ moisture} = 100 \times \left(\frac{m_{\text{wet}} - m_{\text{dry}}}{m_{\text{wet}}}\right)$$

$m_{\text{wet}}$ – mass of wet soil

$m_{\text{dry}}$ – mass of dry soil

3.3.7 Statistical Analyses

ANOVA to determine differences in water color, redox potential, respiration, denitrification, or nosZ gene copy number or relative abundance between treatments were conducted in SigmaStat, Version 3.5 (Systat Software, Inc., 2006). If needed, the data
were natural log transformed before analysis for two-way and three-way ANOVAs to meet homogeneity of variance requirements. For one-way ANOVAs, the analysis was conducted on ranks if homogeneity of variance requirements were not met. TRFLP profiles were compared between sample types (i.e., leaf and soil), date (i.e., day 30 and day 31), and microcosm treatments (i.e., pH and nitrogen addition) with the nonmetric multidimensional scaling (NMS) ordination technique (PC-ORD, Version 5.0; Bruce McCune and MJM Software, 1999). We qualitatively determined whether treatment, sample type, or date affected community structure based on how the pools were clustered on the resulting NMS graphs. Multi-response permutation procedure (MRPP) was performed in PC-ORD to determine if any clustering we determined qualitatively also showed statistical differences between pools based on their community structure. Richness and diversity of each microbial community (bacteria, fungi, and denitrifying microorganisms) based on TRFLP profiles were also assessed in PC-ORD. Graphs were made in SigmaPlot, Version 10.0 (Systat Software, Inc., 2006).

3.4 Results

3.4.1 Water Chemistry

The pH in the microcosms was kept constant throughout the study (Table 3.2). Between day 4 and day 8, the pH for each treatment stabilized and by day 29 were close to the desired pH for each treatment. On day 32, the measured pH values were altered due to the implementation of the nitrogen treatments, but by day 44 returned to values near the target for each treatment and remained stable throughout the remainder of the study (Table 3.2). The % luminance on day 29 of the study increased as the pH decreased, indicating darker colored water in the pH 8 microcosms compared to the pH 5
microcosms (Table 3.3). Redox potential was also inversely related to pH, with the pH 8 microcosms having the most reducing environment (Table 3.4). On days 30 and 31, when the microcosms were pumped with N\textsubscript{2} to make them anoxic, the redox potential was lower than on other dates, but still negatively related to pH (Table 3.4).

### 3.4.2 Ecosystem Functions

The \textit{in situ} respiration rate differed significantly between the pH treatments and over time and the pH x time interaction was also significant (Figure 3.1). The pH 7 and pH 8 microcosms showed little CO\textsubscript{2} efflux on day 1 and ended with a higher respiration than the pH 5 and pH 6 microcosms on day 122 (Figure 3.1). On day 29, the respiration rate was similar between all microcosms, indicating that the microbial communities had recovered from any disturbance associated with the initial pH manipulations (Figure 3.1).

This was especially true for the pH 7 and pH 8 microcosms which had very low respiration on day 1 (and day 4 for the pH 8 microcosms). The pH 7 and pH 8 microcosms were also the most different from their natural pH. Therefore, the nitrogen additions were made on day 29.

When respiration rates were compared with a two-way ANOVA between pH and nitrogen treatments on day 30 and day 31, significant effects of nitrogen treatment were found on both day 30 (p < 0.001) and day 31 (p<0.001) and significant pH x nitrogen treatment interactions were also found on both dates (p = 0.002 for day 30 and p = 0.010 for day 31). On day 30 and day 31, respiration tended to increase with pH and tended to be higher in the $+\text{NH}_4^+$ treatment compared to the $+\text{NO}_3^-$ treatment (Figure 3.2).

The denitrification rates reported here were calculated using the 1 hour / 12 hour gas samples for day 30 and the 24 hour / 40 hour gas samples for day 31, but were similar
to the rates calculated using the N₂O concentrations from the other time points. Data from day 30 and day 31 were analyzed separately because the microcosms were opened up on day 30, following gas sampling, to take microbial community samples. Denitrification rates were markedly higher in the +NO₃⁻ treatment, compared to the +NH₄⁺ and –N treatments where denitrification was not detected on either day 30 or day 31 (Figure 3.3). Within the +NO₃⁻ treatment, there was a positive trend between pH and denitrification rate on day 30 (Figure 3.3). However, this trend was not significant, indicating that pH had little effect on the denitrification rate when NO₃⁻ was available.

The litter in the litter bags increased in weight (Table 3.5), indicating biofilm formation. Therefore, decomposition was not detected in our microcosm study, likely because of low C mineralization under anoxic conditions.

### 3.4.3 Microbial Community Structure

The richness, diversity, and evenness of the bacterial, fungal, and denitrifying communities were consistent across pH and nitrogen treatments (Figure 3.4). The community structure, determined using NMS, of bacteria and fungi, however, was different between pH treatments (Figure 3.5 a and b). For bacteria, this was apparent in both soil and leaf samples, but fungi were only affected in leaf samples (Figure 3.5 a and b) and these groups were shown to be significantly different with MRPP (p < 0.001 for soil and leaf bacteria and leaf fungi). The denitrifiers showed slight clustering with pH treatment, with the pH 8 microcosms slightly different from the rest, especially in the leaf litter; however, the grouping was not strong enough to be considered ecologically significant. The addition of NO₃⁻ or NH₄⁺ also had little effect on microbial community structure, even for denitrifiers, which utilize NO₃⁻ as an electron acceptor (Figure 3.5).
There was a weak clustering of denitrifiers in nitrate treatment microcosms, but again, was not considered strong enough to be ecologically meaningful (Figure 3.5 c). The \textit{nosZ} gene copy number, as determined with QPCR, was also unchanged between $+\text{NO}_3^-$, $+\text{NH}_4^+$, and $-\text{N}$ treatments (Figure 3.6 a). There was a general increase in copy number from day 30 to day 31 in microcosms that received some form of nitrogen (either \textit{NO}_3^- or \textit{NH}_4^+), while no such increase was observed in the microcosms in the $-\text{N}$ treatment. However, this trend was not significant, due to high variability in gene copy number and a significant pH x date interaction (Figure 3.6 a). The significant pH x date interaction was due to the fact that there was an increase in \textit{nosZ} copy number between day 30 and day 31 in the pH 5 and 8 microcosms, while the pH 7 microcosms had a lower \textit{nosZ} copy number on day 30 (Figure 3.6 a). No significant differences in \textit{nosZ} copy number were seen between nitrogen treatments when two-way ANOVAs were conducted on data only from day 30 (p-value = 0.089) or day 31 (p-value = 0.553), or within pH treatments (p-values = 0.609, 0.797, 0.649, and 0.464 for pH treatments 5, 6, 7, and 8, respectively). The relative abundance of \textit{nosZ} containing organisms followed a similar pattern, as no significant differences were seen between nitrogen or pH treatments (Figure 3.6 b). Date was marginally significant with a general increase in \textit{nosZ} relative abundance between day 30 and 31 for the pH 5, 6, and 8 microcosms. However, the pH 7 microcosms had lower \textit{nosZ} relative abundance, which explains the significant pH x date interaction (Figure 3.6 b). Thus, our data indicate that the population size and relative abundance of organisms containing \textit{nosZ} in soil was similar with and without \textit{NO}_3^- addition. QPCR did not detect \textit{nosZ} in the leaves, indicating that the leaves held less than 1000 copies of the gene.
3.5 Discussion

3.5.1 Community Structure and Ecosystem Function

The conceptual model outlined in chapter 1 and modeled after Balser et al. (2001) proposes that the relationship between microbial community structure and ecosystem function is context-dependent, with modulators and resources differentially affecting microbial communities and the functions they perform. Indeed, we found pH to broadly influence the community structure of bacterial and fungal communities, as has been shown for vernal pools in nature (Carrino-Kyker and Swanson, 2008; Chapter 2). This also coincides with a study by Rousk et al. (2009) who found bacterial and fungal growth in soil to be influenced by a pH gradient. Contrary to the conceptual model, however, which predicts modulator change to not influence ecosystem function, we found that community respiration was significantly different between pH treatments, even after 122 days of our study. This may be because our measurement of CO$_2$ efflux encompasses a wide range of microorganisms, both prokaryotic and eukaryotic, that are capable of completely oxidizing carbon sources to CO$_2$. Other studies have also found that general and widespread ecosystem processes are influenced by community dynamics, such as productivity by plants (Tilman et al., 1997), decomposition by macro-detritivores (Heemsbergen et al., 2004), soil saprotrophic fungi (Setälä and McLean, 2004), and soil microbes (Griffiths et al., 2004), and community respiration by aquatic microorganisms (McGrady-Steed et al., 1997). McGrady-Steed et al. (1997) and Morin and McGrady-Steed (2004) have shown that CO$_2$ flux in aquatic microcosms is more variable when microbial richness is low. These studies, coupled with our result that community structure of vernal pool microorganisms was correlated with CO$_2$ flux, suggest that
microbial communities may influence overall respiration rate in aquatic habitats. Unlike respiration, litter decomposition, another widespread process, was not detected in our microcosms. The increase in leaf mass over the course of the study, however, suggests a biofilm formation. As seen for vernal pools in nature (Carrino-Kyker and Swanson, 2008), the microbial communities associated with leaf litter and soil were significantly different from one another. Interestingly, this was true for the nosZ-containing organisms even though denitrifiers are not necessarily expected to contribute to C mineralization of leaf litter. This suggests that the environment created by the biofilms on the leaf litter were capable of supporting denitrifiers.

Also consistent with the Balser et al. (2001) model and the model outlined in Chapter 1, we found the addition of NO$_3^-$ to affect the function of a specific bacterial group, as the denitrification rate was high in microcosms that received NO$_3^-$, but not detected in the absence of NO$_3^-$. However, the addition of NO$_3^-$ only affected ecosystem function and not microbial community dynamics, as the community structure, population size, and relative abundance of nosZ-containing denitrifiers were not altered between the nitrogen treatments. This was consistent with a study by Wertz et al. (2006) who found the denitrification rate to stay constant despite decreasing diversity of denitrifiers. Our results were also consistent with a study by Rich and Myrold (2004) who found that differences in soil denitrifying activity between habitats was unrelated to differences in soil denitrifying community structure, as determined by nosZ TRFLP. Interestingly, we found that the +NH$_4^+$ and --N treatments had similar levels of nosZ copy number to the +NO$_3^-$ treatment, even though denitrification was not detected in these microcosms. This result is similar to that found by Kandeler et al. (2009) who documented no reduction in
nosZ gene copy number in natural forest soils following experimental removal of nitrogen, including NO$_3^-$: Ma et al. (2008) also found no difference in nosZ copy number between cultivated soils (including NH$_4^+$ fertilization) and uncultivated soils. Though not significant, we did observe a general increase in nosZ copy number between day 30 and day 31 for the +NO$_3^-$ and +NH$_4^+$ treatments. This suggests there may have been a N limitation that, once removed, allowed the population to grow.

The conceptual model (see Chapter 1 and Balser et al., 2001) suggests that both ecosystem function and community structure of certain functional groups will respond to the removal of a resource limitation. However, we found that higher denitrification rate in microcosms receiving NO$_3^-$ was not accompanied by changes in community structure, population size, or relative abundance of microorganisms containing the nosZ gene. Thus, the NO$_3^-$ addition may have stimulated gene expression, not population growth and the nosZ gene may have up-regulated in our microcosms with the addition of NO$_3^-$: This indicates that in the absence of NO$_3^-$, the denitrifiers in our microcosms may have formed resting spores or could have performed other ecosystem processes to obtain energy when NO$_3^-$ was not available. The latter is more likely because denitrifiers comprise a taxonomically diverse group of organisms that include more than 50 genera and are known to perform other respiratory pathways, including sulfate reduction, and aerobic respiration (reviewed in Knowles, 1982; Paerl and Pinckney, 1996; McArthur, 2006; Philippot and Hallin, 2006). Thus, our results may depart from the Balser et al. (2001) model because denitrifying bacteria are physiologically plastic and, thereby, capable of utilizing other chemical compounds as terminal electron acceptors in respiration. Consequently, the removal of a resource limitation may have less dramatic effects on the
community of organisms containing *nosZ* because NO$_3^-$ is only one of many resources that can be utilized by this group of bacteria.

It is also possible that we saw no differences in denitrifier population size, relative abundance, or community structure because we added acetylene to our microcosms, which inhibited the reduction of N$_2$O to N$_2$ (Balderston *et al.*, 1976; Yoshinari and Knowles, 1976), and profiled the denitrifier community with *nosZ*, the gene which codes for N$_2$O reductase. Acetylene does not inhibit the reduction of NO$_3^-$ or NO$_2^-$ in the denitrification pathway (Balderston *et al.*, 1976; Yoshinari and Knowles, 1976), but is known to inhibit the growth of some *nosZ*-containing organisms that are not complete denitrifiers (e.g., Yoshinari, 1980). Therefore, the conclusions about denitrifier community structure, population size, and relative abundance in our microcosm study reflected denitrifying organisms which contained both the *nosZ* gene and genes involved in other steps of the denitrification pathway that were not inhibited by acetylene (i.e., denitrifiers in the strict sense, Zumft, 1997), but not organisms which use N$_2$O as their sole electron acceptor. It is possible that *nosZ*-containing true denitrifiers in our microcosms were energetically constrained by the addition of acetylene. For example, we saw no effect of nitrogen addition on denitrifier community structure using *nosZ* TRFLP, while others have found NH$_4^+$ concentration to affect the community structure of denitrifiers in soil when using one of the NO$_2^-$ reductase genes, *nirK*, for TRFLP (Avrahami *et al.*, 2002). However, solely using *nirK*, or any other gene involved in the denitrification pathway, still limits analyses of denitrifier community structure due to the fact that only one gene from a multi-gene pathway is studied (Philippot and Hallin,
A method which allows for more genes in the denitrification pathway to be included, such as a metagenomic study, would help to eliminate this bias.

In many past studies on the relationship between microbial communities and ecosystem function, diversity and richness are the community metrics measured (reviewed in Loreau et al., 2002). In our microcosm study, however, the changes in respiration and denitrification were independent of richness and diversity changes. Our results indicate that changes in richness and diversity alone may not be driving changes in ecosystem processes, but that the identity of individual species within the community, including their ability to up-regulate genes, are also important (Wardle, 2002). A variety of studies on the relationship between microbial diversity and ecosystem function have manipulated the microbial community (e.g., McGrady-Steed et al., 1997; Naeem et al., 2000; Wertz et al., 2006), while in our microcosm study, we did not physically alter the microbial community. The lack of richness or diversity changes reported here could be due to the fact that these metrics are often insensitive of environmental change when applied to microbial communities (Morris and Blackwood, 2007). Thus, when studying the relationship between microbial communities and ecosystem function, manipulating diversity may not accurately represent how microbial communities and processes respond to an environmental perturbation. Nevertheless, it is also possible that we saw no changes in richness and diversity due to the technological limitations of TRFLP, which was used in our study and can only include dominant microbial taxa. Future work should attempt to more accurately determine diversity and richness of the microbial community, including functional groups, under differential environmental change when investigating the relationship between microbial communities and ecosystem function. For example,
studies which use a metagenomic approach can more accurately measure microbial richness and diversity, due to the large number of sequences obtained, and allow for the inclusion of functional diversity (Tringe et al., 2005).

3.5.2 Interaction between Modulator and Resource

Though the Balser et al. (2001) model does not include any interactions between modulators and resources, the modulator described here, pH, influences a variety of other environmental properties, including water color and redox potential (Faust and Aly, 1981), and we have predicted that pH can influence resource availability (see Chapter 1). We observed a positive relationship between pH and water color and negative relationship between pH and redox potential, but these changes signify other potentially important environmental changes, including resource alterations. The increased water color could be due to a greater amount of dissolved organic matter (DOM) leaching from the leaves and soil into the water at a higher pH (Andersson et al., 2000). The leaching of these compounds could provide increased carbon sources for heterotrophic microorganisms in vernal pools with a more basic pH. In a study by Batty and Younger (2007), while pH did not have an effect on overall litter decomposition, it did affect the amount of certain ions released from the litter. Further, the redox potential was influenced by pH, which can affect whether resources exist in oxidized or reduced forms (Drever, 1997). Therefore, the modulator pH can affect the both the availability and chemical form of resources.

Because denitrification involves oxidation-reduction reactions and a variety of enzymes that have pH optima, it seems plausible that pH would have had a substantial affect on enzyme function and, therefore, the denitrification rate in our microcosms.
Other studies have documented effects of pH on denitrification rate (Enwall *et al.*, 2005), denitrifier enzyme activity (Cavigelli and Robertson, 2000) and denitrifying communities (Deiglmayr *et al.*, 2004). Though not significant, we did see an increase in denitrification rate with increasing pH on day 30. It is plausible that this trend is because some denitrifying enzymes have maximum activity at a neutral to slightly basic pH (e.g., Kristjansson and Hollocher, 1981; Sato *et al.*, 1999; Antipov *et al.*, 2005). However, our results are more in concert with Šimek *et al.* (2002) who argued that there is no optimum pH for denitrification. Rather, denitrifiers are likely adapted to a range of environmental pH values by regulating their internal pH much the way that is done for other microorganisms (Booth, 1985; McArthur, 2006). This, in turn, would allow for denitrification to take place at a much wider pH range in nature than suggested by the pH optimum of denitrification enzymes. Therefore, we did not see the interaction between modulator and resource that we expected. We did, however, find an interaction between pH and nitrogen treatments with community respiration, with the highest values tending to be in the +NH$_4^+$ microcosms, suggesting that the microcosms were N-limited. Along with the increase in *nosZ* copy number and relative abundance seen between day 30 and 31 in the +NO$_3^-$ and +NH$_4^+$ microcosms (and all microcosms for relative abundance), these results suggest that resources (i.e., electron acceptors) also eliminate nutrient limitation, making the proposed conceptual model (Balser *et al.*, 2001; Chapter 1) more complex than originally thought.

### 3.6 Conclusions

Overall, our results suggest that the relationship between microbial community structure and ecosystem function is highly context-dependent, which lends some support for the
conceptual models proposed in Chapter 1 and by Balser et al. (2001) and suggests that microbial community stability and its effects on ecosystem function are influenced by the nature of the environmental perturbation under study. Our study provides evidence that organisms within the taxonomically diverse group of denitrifiers maintain a high level of physiological plasticity, which complicates the conclusions drawn based on the conceptual model (i.e., the biology of denitrifiers, and of functional groups in general, may be more complex than suggested by the model). Further, the biogeochemistry of vernal pools may be more complex than suggested by the model, as our results suggest nutrient limitation to be important for microbial respiration and growth. Though modulators and resources offer an interesting paradigm within which to study the relationship between microbial community structure and ecosystem function, they may be oversimplifying environmental change and how microorganisms respond.
Table 3.1 The 4x3 treatment design for the microcosm study.

<table>
<thead>
<tr>
<th>Nitrogen Treatment$^a$</th>
<th>pH Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>+ NO$_3^-$</td>
<td>n=6</td>
</tr>
<tr>
<td>+ NH$_4^+$</td>
<td>n=6</td>
</tr>
<tr>
<td>-- N</td>
<td>n=6</td>
</tr>
</tbody>
</table>

$^a$The nitrogen treatments included nitrate added in a concentration of 10 mg/L NO$_3^-$-N (+NO$_3^-$), ammonium added in a concentration of 10 mg/L NH$_4^+$-N (+NH$_4^+$), and no excess nitrogen added (--N).
<table>
<thead>
<tr>
<th>Day</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.78 ± 0.054</td>
<td>5.33 ± 0.083</td>
<td>7.76 ± 0.19</td>
<td>9.25 ± 0.17</td>
</tr>
<tr>
<td>4</td>
<td>5.03 ± 0.15</td>
<td>5.99 ± 0.12</td>
<td>7.21 ± 0.20</td>
<td>8.87 ± 0.17</td>
</tr>
<tr>
<td>8</td>
<td>5.25 ± 0.14</td>
<td>6.04 ± 0.14</td>
<td>6.85 ± 0.15</td>
<td>7.47 ± 0.17</td>
</tr>
<tr>
<td>15</td>
<td>5.51 ± 0.12</td>
<td>6.19 ± 0.17</td>
<td>6.92 ± 0.16</td>
<td>7.56 ± 0.19</td>
</tr>
<tr>
<td>22</td>
<td>5.20 ± 0.20</td>
<td>6.25 ± 0.19</td>
<td>6.86 ± 0.094</td>
<td>7.58 ± 0.16</td>
</tr>
<tr>
<td>29</td>
<td>5.19 ± 0.25</td>
<td>6.18 ± 0.22</td>
<td>6.99 ± 0.12</td>
<td>7.58 ± 0.16</td>
</tr>
<tr>
<td>32a</td>
<td><strong>5.85 ± 0.54</strong></td>
<td><strong>6.19 ± 0.70</strong></td>
<td><strong>7.07 ± 0.30</strong></td>
<td><strong>7.50 ± 0.46</strong></td>
</tr>
<tr>
<td>44</td>
<td>5.16 ± 0.40</td>
<td>6.16 ± 0.28</td>
<td>7.13 ± 0.11</td>
<td>7.82 ± 0.14</td>
</tr>
<tr>
<td>94</td>
<td>5.25 ± 0.34</td>
<td>6.14 ± 0.17</td>
<td>6.97 ± 0.14</td>
<td>7.71 ± 0.057</td>
</tr>
<tr>
<td>122</td>
<td>5.13 ± 0.24</td>
<td>6.10 ± 0.096</td>
<td>6.93 ± 0.069</td>
<td>7.74 ± 0.097</td>
</tr>
</tbody>
</table>

Mean 5.21 ± 0.09 6.05 ± 0.08 7.06 ± 0.09 7.91 ± 0.20

*The pH values for day 32 (in bold face) were soon after the nitrogen treatments were implemented on day 29 and could explain the rather high increase in average pH for the pH 5 treatment.
Table 3.3 Water color (mean ± standard error) of the microcosms measured on day 28 in a sub-set of the microcosms (n=3)

<table>
<thead>
<tr>
<th>pH Treatment</th>
<th>Water Color (% luminance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>92.3 ± 0.006\textsuperscript{a}</td>
</tr>
<tr>
<td>6</td>
<td>62.9 ± 0.04\textsuperscript{b}</td>
</tr>
<tr>
<td>7</td>
<td>11.6 ± 0.02\textsuperscript{c}</td>
</tr>
<tr>
<td>8</td>
<td>2.5 ± 0.003\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{p-value} <0.001

*p-value obtained from a one-way ANOVA with significance set at (α = 0.05). Statistically different values are indicated with different superscript letters. Because these data were taken prior to the nitrogen additions, only differences between pH values were determined.
Table 3.4 Redox potential (mean ± standard error) monitored throughout the study.

<table>
<thead>
<tr>
<th>pH Treatment</th>
<th>Redox Potential (relative mV)</th>
<th>pH Treatment</th>
<th>Redox Potential (relative mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td>Day 29</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>269.78 ± 4.23^a</td>
<td>5</td>
<td>189.37 ± 11.21^a</td>
</tr>
<tr>
<td>6</td>
<td>212.41 ± 12.16^ab</td>
<td>6</td>
<td>65.85 ± 8.97^b</td>
</tr>
<tr>
<td>7</td>
<td>111.59 ± 6.87^bc</td>
<td>7</td>
<td>-18.62 ± 4.94^b</td>
</tr>
<tr>
<td>8</td>
<td>69.91 ± 3.67^c</td>
<td>8</td>
<td>-99.99 ± 2.88^c</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>p-value</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>Day 30</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>191.69 ± 8.30^a</td>
<td>5</td>
<td>17.14 ± 14.72^a</td>
</tr>
<tr>
<td>6</td>
<td>72.89 ± 5.41^b</td>
<td>6</td>
<td>-52.65 ± 11.13^b</td>
</tr>
<tr>
<td>7</td>
<td>-27.82 ± 1.74^c</td>
<td>7</td>
<td>-111.47 ± 7.63^c</td>
</tr>
<tr>
<td>8</td>
<td>-101.88 ± 5.35^d</td>
<td>8</td>
<td>-157.85 ± 4.70^d</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>p-value</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td>Day 31</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>84.57 ± 6.44^a</td>
<td>5</td>
<td>-7.43 ± 11.36^a</td>
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<tr>
<td>6</td>
<td>18.82 ± 4.49^b</td>
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<td>-71.86 ± 10.09^b</td>
</tr>
<tr>
<td>7</td>
<td>-28.00 ± 2.48^b</td>
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<td>-118.93 ± 7.49^c</td>
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<td>8</td>
<td>-75.87 ± 2.10^c</td>
<td>8</td>
<td>-165.77 ± 5.09^d</td>
</tr>
<tr>
<td>p-value</td>
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<td>p-value</td>
<td>&lt;0.001</td>
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<tr>
<td>Day 15</td>
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<td>Day 94</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>65.71 ± 3.67^a</td>
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<td>152.63 ± 21.65^a</td>
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<tr>
<td>6</td>
<td>12.11 ± 4.53^b</td>
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<td>20.02 ± 11.83^a</td>
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<td>7</td>
<td>-41.90 ± 3.00^c</td>
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<td>8</td>
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<td>p-value</td>
<td>&lt;0.001</td>
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<tr>
<td>Day 22</td>
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<td>Day 122</td>
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</tr>
<tr>
<td>5</td>
<td>150.23 ± 8.70^a</td>
<td>5</td>
<td>146.37 ± 8.63^a</td>
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<td>31.23 ± 6.68^b</td>
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<td>44.76 ± 7.87^b</td>
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<td>-34.06 ± 3.32^b</td>
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<td>-35.91 ± 6.23^c</td>
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<td>8</td>
<td>-93.44 ± 2.94^c</td>
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<td>-96.23 ± 5.38^d</td>
</tr>
<tr>
<td>p-value</td>
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<td>p-value</td>
<td>&lt;0.001</td>
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</tbody>
</table>

p-value obtained from a one-way ANOVA to determine difference between pH treatments on each day. The significance was set at (α = 0.05). Different superscript letters indicate statistically different values for each measurement within one day. ND = not detected.
Table 3.5 Percent mass remaining of beech leaf punches in litter bags following four months in the microcosm study.

<table>
<thead>
<tr>
<th>Microcosm</th>
<th>% Mass Remaining</th>
<th>Microcosm</th>
<th>% Mass Remaining</th>
<th>Microcosm</th>
<th>% Mass Remaining</th>
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<td>105</td>
<td>49</td>
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</tbody>
</table>
Figure 3.1 *In situ* community respiration, measured as CO$_2$ efflux from the microcosms (mean ± standard error), over the course of the study. The pH treatments are represented by different colors and shapes (see figure legend). A two-way ANOVA, using time as a repeated factor and pH, was conducted starting on day 8 (when respiration was detected in all microcosms). The p-values are shown and significant values ($\alpha = 0.05$) are indicated with an *. The data were natural log transformed prior to statistical analysis.
Figure 3.2 In situ community respiration, measured as CO₂ efflux from the microcosms, for each pH and nitrogen treatment on days 30 and 31 (mean with error bars showing standard error). The pH treatments are represented by different colors (see figure legend). A two-way ANOVA, using pH and nitrogen treatments as factors was conducted and the p-values are shown with significant values (α = 0.05) indicated with an *. The data were natural log transformed prior to statistical analysis.
Figure 3.3 Denitrification rate (mean with error bars showing standard error) for each treatment group. The rates for the +NH$_4^+$ and --N treatments were below the level of detection of our method (indicated by “ND”). One-way ANOVAs were conducted within each +NO$_3^-$ treatment to determine differences with pH and p-values are shown. A Kruskal-Wallis ANOVA on ranks was used for the day 31 data because these data failed the equal variance test. The pH treatments are represented by different colors (see figure legend).
**Figure 3.4 (previous page)** Mean (± standard error) richness (closed symbols, solid lines) and Shannon-Wiener diversity (open symbols, dotted lines) for the a) bacterial, b) fungal, and c) denitrifier communities as determined by Terminal Restriction Fragment Length Polymorphism (TRFLP). The restriction enzymes used for the TRFLP profile comparisons shown here were *MspI*, *HaeIII*, and *TaqI* for the communities of a) bacteria, b) fungi, and c) denitrifying organisms, respectively. The other restriction enzymes used (see Experimental Procedures section) produced similar results. The pH treatments are indicated by different shapes and colors (see legend).
2-dimensional solution
stress = 12.45%

3-dimensional solution
stress = 14.26%

3-dimensional solution
stress = 12.85%
Figure 3.5 (previous page) Nonmetric multidimensional scaling ordination graphs showing the a) bacterial, b) fungal, and c) denitrifier community structures as determined by Terminal Restriction Fragment Length Polymorphism (TRFLP). The stress value for each n-dimensional solution is shown. The restriction enzymes used for the TRFLP profile comparisons shown here were *MspI*, *HaeIII*, and *TaqI* for the communities of a) bacteria, b) fungi, and c) denitrifying organisms, respectively. The other restriction enzymes used (see Experimental Procedures section) produced similar results. Closed symbols represent soil samples, while crossed symbols represent leaf samples. The pH treatments are indicated by different colors (see legend). Different nitrogen treatments are shown by symbol shape with squares representing +NO$_3^-$, diamonds representing +NH$_4^+$, and circles representing --N. The two sample dates (day 30 and day 31) are not indicated on the graphs, but no differences in community structure were seen between dates.
Size (a) and relative abundance (b) of the denitrifier community in soil (mean with error bars showing standard error) estimated by nosZ gene copy number for each treatment group. The pH treatments are represented by different colors (see figure legend). The p-values from a three-way ANOVA, including nitrogen treatment, pH, and date as factors, are given. Significant p-values ($\alpha = 0.05$) are indicated with an *. Data were natural log transformed prior to the ANOVA.

**Figure 3.6 (previous page)** Size (a) and relative abundance (b) of the denitrifier community in soil (mean with error bars showing standard error) estimated by nosZ gene copy number for each treatment group. The pH treatments are represented by different colors (see figure legend). The p-values from a three-way ANOVA, including nitrogen treatment, pH, and date as factors, are given. Significant p-values ($\alpha = 0.05$) are indicated with an *. Data were natural log transformed prior to the ANOVA.
Chapter 4

Metagenomic Analysis of Microbial Community Structure and Metabolic Diversity in Experimental Vernal Pools Subject to Different Levels of Nitrate Input.

4.1 Summary

Because of the influence microbes have on carbon and nutrient cycling, many current studies focus on understanding the relationship between microbial community structure and metabolic processes that control ecosystem processes. Vernal pools are ideal systems within which to study this relationship because abiotic conditions fluctuate rapidly and lead to a spatially and temporally dynamic microbial community. Our previous work on vernal pools suggested that a difference in ecosystem function (e.g., denitrification) does not necessarily translate to differences in microbial community structure. Consequently, we hypothesized that either 1) microbial communities contain a large number of taxa that possess metabolic flexibility and can quickly respond to changes in resource availability without changes in community structure or 2) standard PCR-based techniques used to explore microbial community structure limit our perception of taxonomic diversity and abundance and lead to erroneous conclusions about the relationship between microbial richness, diversity, and community structure and metabolic function. Here, we report the sequencing of two metagenomes to investigate vernal pool microbial communities from microcosms that were either enriched with nitrate (NO$_3^-$) and had a high denitrification rate or were un-enriched with NO$_3^-$ (no detected denitrification). Even though the metagenomes consisted of over 7,000 bacterial environmental gene tags (EGTs) each, no difference in bacterial richness or diversity were found between them, supporting our previous conclusion that changes
in ecosystem processes can be independent of richness or diversity changes. Coupled with the finding that the metagenomes contained EGTs matching a variety of metabolic pathways, including aerobic respiration, sulfate reduction, fermentation, and methanogenesis, these results suggest that microbes, including denitrifiers, may rely on a high level of metabolic flexibility for survival and growth in the absence of certain resources. However, changes in proportional representation of some bacterial EGTs, specifically in the groups *Acidobacteria* and *Alphaproteobacteria*, were altered with NO$_3^-$ addition, indicating that standard PCR-based techniques may not provide a large enough number of DNA fragments to detect changes in microbial community composition. Further, a large number of EGTs matched with saprotrophic fungi and bacterial decomposer sequences, as well as sequences for organic matter degradation, suggesting the importance of decomposition in vernal pools. The finding of EGTs that matched with genes for antibiotic production and antibiotic resistance suggests competition between fungal and bacterial decomposers for organic matter. Metagenomic analysis of microbial communities from a sample of vernal pool microcosms indicates that these habitats may represent unique sites of microbial metabolic diversity and carbon and nutrient cycling in forest ecosystems.

4.2 Introduction

Because microorganisms exert a strong control on carbon and nutrient cycling in natural environments, an emerging area in microbial ecology seeks to make connections between the structure and function of microbial communities. A variety of studies have shown a relationship between microbial diversity and a variety of ecosystem processes, including CO$_2$ flux (McGrady-Steed *et al.*, 1997), primary productivity (Cardinale *et al.*, 2006), and
decomposition (Griffiths et al., 2004). Others have contended that microbial diversity is instrumental in maintaining ecosystem reliability (Naeem and Li, 1997) and predictability (Morin and McGrady-Steed, 2004). These studies, however, have investigated widespread ecosystem processes that are carried out by many taxa or have measured ecosystem characteristics that encompass many microorganisms in the community. Such relationships between diversity and ecosystem function, however, may not be apparent when a process is performed by a functional group, such as denitrification (Wertz et al., 2006; Chapter 3). In the previous chapter, we reported the results of a study that examined the relationship between microbial community structure and function in experimental vernal pool microcosms. We found that overall microbial community structure was unaffected by the addition of a potential limiting resource, nitrate (NO$_3^-$), although large increases in denitrification were observed. Consequently, we hypothesized that either 1) microbial communities contain a large number of taxa that possess metabolic flexibility and can quickly respond to changes in resource availability without changes in community structure or 2) standard PCR-based techniques used to explore microbial community structure limit our perception of taxonomic diversity and abundance, and lead to erroneous conclusions about the relationship between community richness, diversity, and structure and metabolic function.

Because the concentration and availability of chemical compounds fluctuate in the environment, many microorganisms possess tremendous metabolic flexibility. Many microorganisms are facultative, meaning that they are capable of switching between different compounds used for energy generation during respiration. For example, microbes in the genus *Pseudomonas* are known for utilizing a wide variety of organic
compounds for carbon and energy with some species using over 100 different compounds and only a few known species using less than 20 and are known to be involved in anaerobic respiration (Madigan et al., 2003). Functional groups, which use a variety of inorganic and organic compounds as electron acceptors or donors during respiration, also display a high degree of metabolic flexibility. Sulfate reducing bacteria, for example, use sulfate as their terminal electron acceptor, but are capable of using a range of electron donors, such as lactate, pyruvate, malate, sulfonate, and certain alcohols (Madigan et al., 2003). Further, some denitrifying bacteria are known to perform other respiratory pathways, including aerobic respiration and the anaerobic pathway of sulfate reduction (reviewed in Knowles, 1982; Paerl and Pinckney, 1996; McArthur, 2006; Philippot and Hallin, 2006). This metabolic diversity is expected given the unpredictable nature of the environment; however, current ecological models often ignore this flexibility, which is partly due to the methods which are used to investigate these microbial functional groups.

Though PCR-based approaches used to profile microbial functional groups have made significant advances in characterizing these groups of organisms, they provide a limited view of microbial diversity due to the fact that only one functional group can be profiled at a time. This is further limited by the fact that organisms from functional groups often possess more than one gene involved in the processes they perform, but individual PCR reactions can only amplify one gene from the pathway. For example, denitrification, which is the reduction of nitrate (NO₃⁻) to nitrogen gas (N₂), involves seven enzymes (Figure 4.1), the genes for which are currently used to investigate denitrifiers with PCR-based approaches (Bothe et al., 2000; Philippot and Hallin, 2006). Using environmental genomic techniques, denitrifying communities have been profiled in
soil, rhizosphere, and aquatic habitats (see Bothe et al., 2000 and Philippot and Hallin, 2006 for reviews). These studies have made substantial contributions toward understanding the distribution of denitrifying organisms in the natural world and characterizing the relationship between denitrifier diversity and function. However, the methods used for profiling denitrifier communities have characterized one gene region at a time from a multi-gene pathway (e.g., Avrahami et al., 2002; Deiglmayr et al., 2004; Rich and Myrold, 2004; Enwall et al., 2005; Kandeler et al., 2009; Chapter 3). This challenges the ability to draw conclusions about denitrifiers because the PCR primers only amplify a subset of the denitrifying community (reviewed in Philippot and Hallin, 2006). For example, some denitrifiers are known to possess only one of the two nitrite reductases, NirS or NirK, while others lack nitrous oxide reductase (Bothe et al., 2000; Philippot and Hallin, 2006). Thus, studies where only the nirS, nirK, or nosZ genes are used to profile the denitrifying community are known to eliminate some organisms from analyses and current PCR-based approaches can underestimate the diversity of denitrifier communities that can be detected in soil (Rösch and Bothe, 2009).

Alternatively, metagenomic studies involve analysis of whole community microbial genomes, which allows for microbes to be studied irrespective of their culturability and taxonomic identities (e.g., Venter et al., 2004; Tringe et al., 2005; Edwards et al., 2006). Metagenomics further allows for both phylogenetic diversity and potential functional capabilities of microbes to be obtained from one sequencing effort (Meyer et al., 2008). Here, we utilized a metagenomic approach to profile the taxonomic and metabolic diversity of microbial communities in vernal pool microcosms and investigate the relationship between denitrification and microbial diversity. Two
metagenomes were created, one for replicate microcosms that displayed a high level of
denitrification following NO$_3^-$ addition and one for replicate microcosms where NO$_3^-$ was
not added and denitrification was not detected. Because all regions of the microbial
genomes were randomly targeted, these methods allowed for the potential inclusion of all
denitrifying genes and, thus, an in depth characterization of the relationship between
microbial richness, diversity, and community composition and an ecosystem process.

4.3 Materials and Methods

4.3.1 Sample Preparation

Vernal pools were replicated in 50 mL microcosms and subjected to nitrogen addition
and pH treatments (see Chapter 3 for details of the experimental set up). The microcosms
used for the metagenomic analysis were those that were manipulated to a pH around 6
and received either an addition of 10 mg NO$_3^-$-N or an equal volume of de-ionized water
as a control. The NO$_3^-$ addition and control treatments were used because denitrification
rate differed in these microcosms (Chapter 3). Only microcosms held at a constant pH of
around 6 were used because they closest to the ambient soil pH. DNA was extracted
from soil samples collected from these microcosms on D30 (as described in Chapter 3)
and used to create two metagenomes: one for the NO$_3^-$ treatment (labeled +NO$_3^-$) and one
for the control treatment (labeled --N). There were three replicate DNA samples from the
+NO$_3^-$ microcosms and three replicates from the --N microcosms.

4.3.2 454 Pyrosequencing

DNA was amplified with the illusa Genomi phi V2 amplification kit (GE Healthcare
Life Sciences, Inc., Piscataway, NJ) by following the manufacturer’s protocol. The
amplified DNA was shown to be strictly from the microcosm samples by the inclusion of
a negative control, which contained distilled water along with the Genomiphi reagents. Two replicate Genomiphi reactions were prepared for each microcosm DNA sample, making six reactions total for each treatment (three replicate microcosm DNA samples x two replicate Genomiphi reactions). The Genomiphi reactions randomly amplified regions of genomic DNA using primers of random sequences. For our microcosms, then, the 8 µg of amplified DNA from the +NO$_3$ sample and the 10 µg of amplified DNA from the –N sample potentially included segments of DNA from all microbial species in the soil sample and from regions throughout the microbial genomes. The amplified DNA from Genomiphi reactions was precipitated with sodium acetate and purified with 80% cold ethanol before being sent to Inqaba Biotec (Pretoria, South Africa) for 454 pyrosequencing on a GS-FLX platform.

4.3.3 Sequence Analysis

Because the metagenomes constructed from our microcosms contained DNA reads from multiple species, they were analyzed unassembled. Thus, each DNA fragment that was characterized represented an environmental gene tag (EGT), or a short segment of a gene found in the microcosm samples. The metagenomes were uploaded to the Meta Genome Rapid Annotation of Sequence Technology (MG-RAST) server (Meyer et al., 2008) where they were analyzed for taxonomic identity and functional gene content using a BLASTx comparison to the SEED database (Overbeek et al., 2004). The SEED database includes hundreds of complete and partial genomes of many eukaryotes, Bacteria, Archaea, and viruses (Overbeek et al., 2004), which allowed for both a phylogenetic and metabolic profile of our metagenomes by matching the EGTs to taxa and subsystems, respectively (Figure 4.2). Matching with subsystems allowed for predicted functions to
be determined. For example, if EGTs for nitrite reductase, nitric oxide reductase, and/or nitrous oxide reductase were found in a metagenomic data set, denitrification was a predicted function of the microorganisms profiled. The process of denitrification, along with other microbial processes that use nitrogenous compounds, were further categorized in the MG-RAST server as types of nitrogen metabolism. The phylogenetic profile also involved hierarchical categories ranging from the organismal to domain level. Within the MG-RAST server, we compared the proportional abundance of taxa or subsystems at several hierarchical levels between the two metagenomes (Figure 4.2). Only matches that had an e-value of \(10^{-5}\) or lower and had sequence similarity of 50 base pairs or greater were included (as in Yergeau et al., 2010). These values are more stringent than the default settings for MG-RAST and are considered the minimum e-value and base pair matches for rDNA matches (Meyer et al., 2008). This yielded sequences matches of over 5,000 metabolic EGTs and over 9,000 phylogenetic EGTs for the metagenomes (Table 4.1). Richness and Shannon and Simpson’s diversity indices of bacterial taxa in each metagenome were calculated following the formulas in McCune and Grace (2002) and using the organismal (i.e., species) level in MG-RAST level (which was the lowest hierarchical category) as the cutoff. Graphs were made in SigmaPlot Version 10.0 (Systat Software, Inc., 2006).

4.4 Results

4.4.1 Metagenome Comparison

Though the microcosms from which the metagenomes were constructed showed a large difference in denitrification rate (see Chapter 3), the bacterial diversity was similar (Table 4.2). Five denitrification EGTs were detected in our metagenomes included nitric oxide
reductase and nitrous oxide reductase (Table 4.3) and the proportional representation of these EGTs were higher in the –N treatment where denitrification rate was not detected (Figure 4.3). However, because the number of denitrification EGTs was low, this suggests perhaps a low abundance of these organisms in the microcosms. Interestingly, the –N metagenome had a higher relative number of EGTs matching with genes involved in ammonification and ammonia assimilation, and nitrosative stress EGTs were only present in the –N metagenome (Figure 4.3). EGTs for nitric oxide synthase had a higher proportional representation in the +NO₃⁻ metagenome (Figure 4.3).

### 4.4.2 Metabolic Diversity

A number of subsystem sequences were found in both the +NO₃⁻ and the –N metagenomes, except for an EGT associated with dormancy and sporulation, which was only found in the –N metagenome (Figure 4.4). Both metagenomes, however, did contain EGTs that matched with a diversity of microbial metabolic genes, including those involved in catabolic reactions of sulfur and nitrogen metabolism, respiration (including both aerobic and anaerobic respiratory pathways), and the catabolism of aromatic compounds (Figure 4.4). Within the carbohydrates subsystem, EGT matches to methanogenesis and fermentation were found, again showing the metabolic diversity present in the metagenomes (Figure 4.5 a).

### 4.4.3 Phylogenetic Diversity

The majority of EGTs in both the +NO₃⁻ and the –N metagenomes matched with bacterial and eukaryotic sequences of the SEED database (Figure 4.6). The bacterial EGTs included representatives from a number of groups known to be involved in a wide array of metabolisms, again indicating the wide array of metabolic diversity detected in the
microcosms. These metabolically diverse groups included Chlorobi, or green sulfur bacteria which are photoautotrophs, Chloroflexi, or green non-sulfur bacteria, which includes photoheterotrophs, Firmicutes, which includes some fermenters in the class Clostridia, the oxygenic phototrophs of Cyanobacteria, and the predominantly anaerobic chemotrophs of Spirochaetes (Figure 4.7 a).

The bacterial EGTs also had high representation of Proteobacteria, Bacteriodetes, and Acidobacteria (Figure 4.7 a), which are commonly found in soil and aquatic samples. Interestingly, the +NO\textsubscript{3} metagenome had a markedly higher proportional representation of Acidobacteria and Alphaproteobacteria (Figure 4.7). The large difference in Acidobacteria proportional representation is of special interest because this group of organisms was discovered with the advent of molecular methods and has only recently been cultured. Other bacterial sequences found in the metagenomes were from groups tolerant of high levels of environmental change, such as Aquificae, which thrive in harsh, high temperature environments, Deinococcus-Thermus, which are resistant to environmental hazards, and Firmicutes, many of which produce endospores to survive desiccation (Figure 4.7 a).

4.4.4 Decomposer EGTs

A number of EGTs were found to match with genes involved in organic matter degradation processes, including hexitol degradation, inositol catabolism, and utilization of other sugars classified under the sugar alcohols category in the carbohydrates subsystem (Figure 4.5 a). Subsystems involved in organic matter decomposition were also found under the amino acids category, which included several catabolic reactions, such as glycine and serine utilization, histidine utilization, and the degradation of several
amino acids (Figure 4.5b) and the protein metabolism category, which included EGTs involved in protein degradation. The phylogenetic profile also indicated the importance of decomposition in vernal pools as many decomposer taxa were found, including *Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Alphaproteobacteria*, and *Betaproteobacteria* (Figure 4.7). Most striking, though, was that among eukaryotic EGTs in both metagenomes, fungi had the highest proportional representation and were dominated by Ascomycota, a group which contains saprotrophic fungi (Figure 4.8). EGTs that matched with fungal cytostatics (classified under the secondary metabolism subsystem), biosynthesis of chorismate, an intermediate in antibiotic synthesis (classified under amino acids), and prokaryotic antibiotic resistance (classified under the stress response subsystem) were also found in the metagenomes and suggest competition between bacterial and fungal decomposers.

**4.5 Discussion**

**4.5.1 Nitrogen Metabolism**

The +NO$_3^-$ and −N metagenomes were constructed from microcosms that displayed largely different denitrification rates (see Chapter 3). However, this difference in function was independent of a difference in bacterial richness or diversity as determined with a metagenomic approach. Neither richness nor diversity (Shannon nor Simpson’s) were different between the treatments. This is consistent with previous results from the same microcosms, where no differences in bacterial, fungal, or denitrifier diversity were observed following terminal restriction fragment length polymorphism (TRFLP) profiling (Chapter 3). Similarly, Wertz et al. (2006) found no change in denitrification rate with decreasing bacterial and denitrifier diversity and suggested processes performed by
functional groups, such as denitrification, may not be influenced by community dynamics to the same degree as widespread functions. This could be due to the fact that denitrification can be carried out by a wide variety of taxa found in more than 50 genera (Philipot and Hallin, 2006). This diversity of denitrifiers, including their metabolic flexibility, could also explain the observation in this study of a similar proportional representation of denitrification EGTs between metagenomes. Despite clear differences in denitrification between +NO$_3^-$ and –N microcosms, we previously found no change in denitrifier community structure, population size, or relative abundance using nosZ TRFLP and QPCR (Chapter 3). The results from the metagenomes showed no proportional increase in denitrification EGTs with NO$_3^-$ addition. However, the low number of denitrifier EGTs limits any comparison that can be made between the treatments. Nonetheless, the fact that both nitric oxide reductase and nitrous oxide reductase EGTs were found, at least in the --N metagenome, indicates that this approach is potentially useful for investigating multi-gene pathways.

We previously found a large increase in denitrification with NO$_3^-$ addition without accompanying changes in either denitrifier community structure, population size, or relative abundance and suggested that denitrifiers in our microcosms either formed resting spores or used another form of metabolism for energy in the absence of NO$_3^-$ (Chapter 3). The metagenomic approach used in this study can not exclude either of these possibilities, as a diversity of metabolisms and taxa containing diverse metabolisms were found in both metagenomes and an EGT for dormancy and sporulation was found in the --N metagenome. However, some of these bacteria may be flexible enough to also use other compounds as terminal electron acceptors. For example, denitrifiers have been
shown to perform sulfate reduction (Bursakov et al., 1995) and metabolize aromatic compounds (Trautwein et al., 2008), both functions which were detected in our metagenomic analysis. (These metabolisms, though, provide less free energy than denitrification and, thus, require energy costs for the organisms.) It is therefore likely that denitrification is inducible in many organisms, which respond to increases in NO$_3^-$ by increasing expression of genes involved in the respiratory pathway. Since our metagenome approach targeted DNA, we may have failed to detect changes in gene expression.

The results from other nitrogen metabolism EGTs besides denitrification suggest that this approach can detect meaningful EGT responses to changing environmental conditions. For example, NO$_3^-$ and nitrite ammonification EGTs had a higher proportional representation in the –N treatment. EGTs for nitrosative stress, a process which relieves the stress of NO accumulation during ammonification, were only detected in the –N metagenome, and the relative number of EGTs for ammonia assimilation were markedly higher in the –N metagenome. This suggests that ammonification was taking place in the microcosms that were not amended with NO$_3^-$, which is consistent with Strohm et al. (2007) who have shown that ammonification is preferred as a NO$_3^-$ reducing respiratory process over denitrification when NO$_3^-$ is limiting. The +NO$_3^-$ metagenome had a higher proportional representation of nitric oxide synthase EGTs, likely in response to the build up of N$_2$O as a result of using the acetylene block technique to measure denitrification rate (Groffman et al., 1999; see Chapter 3 for further information on the acetylene block technique). Nitric oxide synthase, in bacteria, is involved in protection against oxidative stress and antibiotics (Gusarov and Nudler, 2005;
Gusarov et al., 2009) and, in plants, creates NO to be used as a signal molecule (Corpas et al., 2006). While no studies have been conducted on the effects of N$_2$O on nitric oxide synthase in plants or microbes, a potential role has been suggested in animal models (Lack Cope et al., 2010).

4.5.2 The Effect of NO$_3^-$ Addition on Microbial Community Structure

The sequencing effort described here showed a markedly higher proportional representation of Alphaproteobacteria and Acidobacteria in the +NO$_3^-$ metagenome. Similarly, using freshwater microcosms, Barlett and Leff (2010) found an increase in Alphaproteobacteria abundance when NO$_3^-$ was present as a nitrogen source and suggest a competitive advantage to this group of organisms under these conditions. Under anoxic conditions, such as our microcosms, higher physiological activity and the ability to uptake substrates have been reported in several species of Alphaproteobacteria only when NO$_3^-$ or NO$_2^-$ were present as an electron acceptor (Kragelund et al., 2006). Therefore, in our microcosms, there could have been a competitive advantage to the Alphaproteobacteria due to greater growth compared to other facultative organisms in an anoxic environment with abundant NO$_3^-$ . The Acidobacteria may also have had a competitive advantage in the +NO$_3^-$ treatment, as cultured strains were recently found to possess NO$_3^-$ and NO$_2^-$ reducing genes (Ward et al., 2009). Ward et al. (2009) also suggest that Acidobacteria are resistant to desiccation and, thus, capable of tolerating fluctuations in soil hydration. This may allow their survival in vernal pool communities and, when NO$_3^-$ becomes readily available, they are able to rapidly utilize this resource. Alphaproteobacteria (Pinhassi and Berman, 2003), and likely Acidobacteria (Ward et al., 2009), are adapted to low nutrient conditions. While this seems counterintuitive to our
microcosm study, vernal pools in nature are known to be oligotrophic (Carrino-Kyker and Swanson, 2007). The *Alphaproteobacteria* and *Acidobacteria* in vernal pools, then, may be adapted to survival in the disturbed, low nutrient conditions of these habitats and only once NO$_3^-$ becomes readily available do they have a competitive advantage due to their growth capabilities in the presence of NO$_3^-$. 

These taxonomic changes were not found in a previous examination of bacteria in these microcosms with TRFLP (Chapter 3). From the TRFLP results, we concluded that an addition of NO$_3^-$, which is utilized by functional groups, did not alter the overall bacterial community structure or the denitrifier functional group (Chapter 3). TRFLP is a coarse community profiling tool, however, and may not be able to detect small changes in bacterial communities. In the current study, metagenome comparison suggested that there may have been small changes in bacterial community structure and the abundance of specific taxa. Because the metagenomes consisted of over 7,000 and 14,000 bacterial EGTs for the +NO$_3^-$ and –N treatments, respectively, this is a result that would not have been reached using more standard PCR-based profiling techniques, which often rely on sequences of a few hundred clones. Therefore, bacterial community structure may, in fact, respond to NO$_3^-$ availability.

### 4.5.3 Microbial Diversity in Vernal Pools

The metabolic and taxonomic diversity uncovered in this metagenomic study provides insight into vernal pool microbial communities. Several groups that were identified in the metagenomes are known for resistance to environmental hazards or survival in extreme environments, including members of *Actinobacteria*, *Firmicutes*, and *Deinococcus-Thermus*, which possess adaptations that allow for desiccation tolerance.
(Battistuzzi and Hedges, 2008). Such adaptations are necessary in vernal pools which become dry in the summer (Colburn, 2004). Our metagenomes also included matches to the phylum *Aquificae*, which have only been found in hot springs (Griffiths and Gupta, 2006). The presence of *Aquificae* in our metagenomes may be due to closely related microorganisms in vernal pools, which, like members of the *Aquificae*, possess thermoregulatory genes to survive the large variations in temperature over the course of only several months (Carrino-Kyker and Swanson, 2007). Other environmental parameters beside temperate fluctuate widely and rapidly over the course of the spring season in vernal pools (Bonner *et al.*, 1997; Colburn, 2004; Carrino-Kyker and Swanson, 2007; Carrino-Kyker and Swanson, 2008). Dissolved oxygen, for example, is near saturation early in the spring season when water temperatures are cold following snowmelt and wind is high to mix the shallow ponds (Carrino-Kyker and Swanson, 2007). The oxygen is reduced to near zero throughout the spring season as water temperatures increase, wind decreases, and aquatic insects, tadpoles, and salamander larvae populate the pools (Bonner *et al.*, 1997; Colburn, 2004; Carrino-Kyker and Swanson, 2007). One way that microorganisms survive fluctuations in dissolved oxygen concentration in aquatic environments is by forming microbial consortia which consist of microbes possessing complementary metabolic pathways (Paerl and Pinckney, 1996). Our metagenomes contained a rather wide array of metabolic EGTs, including those involved in denitrification, nitrogen fixation, sulfate reduction, fermentation, and methanogenesis, which are processes inhibited by oxygen, as well as EGTs involved in both aerobic and anaerobic respiration, suggesting the presence of microbial consortia. The presence of many metabolic pathways could also allow for vernal pool microbial
communities to change in composition, between groups of aerobic, facultative, and anaerobic organisms, over the season as dissolved oxygen concentrations change. In fact, Carrino-Kyker and Swanson (2008) found that bacterial communities were temporally dynamic with different taxa present in April, May, and June as the physical and chemical environment of vernal pools changed over time. Regardless of whether vernal pool microbes form consortia or change over time, these habitats may represent unique sites of metabolic diversity in forest ecosystems.

This metagenomic analysis also suggests that vernal pools are rich in heterotrophic organisms, including saprotrophic fungi, as Ascomycota dominated the eukaryotic EGTs in both metagenomes. This is consistent with previous studies where denaturing gradient gel electrophoresis revealed an abundance of Ascomycota sequences in natural vernal pools (Carrino-Kyker and Swanson, 2008; Chapter 2). The presence of Alphaproteobacteria and Betaproteobacteria in the metagenomes is consistent with Carrino-Kyker and Swanson (2008) who found high numbers of sequences matching to these groups in natural vernal pools. Similarly, Olapade and Leff (2004) found that these groups dominated bacterial biofilm assemblages in a stream and Ibekwe et al. (2007) observed an abundance of Betaproteobacteria in the soil and rhizosphere of a wetland. These authors have suggested that Alphaproteobacteria and Betaproteobacteria may be important in aquatic systems for the degradation of organic matter. Alphaproteobacteria, Actinobacteria, and Planctomycetes, which are other groups of bacteria found in our metagenomes, were also found in high numbers in peat bogs during decomposition of Sphagnum and Firmicutes and Bacteroidetes are believed to be important decomposers in wetlands (Kulichevskaya et al., 2006). The presence of EGTs involved in protein, amino
acid, and sugar degradation in the metagenomes further suggests the importance of organic matter degradation for vernal pool microbial growth. Allochthonous leaf litter input, which has been quantified at 133 g/m² for one year in a wooded vernal pool in Ontario (Bärlocher et al., 1978), is an important source of carbon for fungi and bacteria. This carbon, however, may not be readily available for vernal pool microbes during wet phases due to hypoxic conditions that limit leaf litter decomposition (Chapter 3). Bacteria engage in interspecific competitive behaviors, such as general antibiotic production, more readily when carbon is limiting (Garbeva and de Boer, 2009), which could explain the presence of the chorismate and antibiotic resistance EGTs in the metagenomes. Both the antibiotic resistance and chorismate matches were composed of bacterial sequences, suggesting that vernal pool bacteria compete for limiting carbon and nutrients. A match to fungal cytostatics, classified as biologically active compounds used in fungal defense, were also found in our metagenomes indicating that bacteria may also compete with fungi in vernal pools. Competition between fungi and bacteria for organic carbon has been documented in both soil and aquatic habitats (Wohl and McArther, 2001; Rousk et al., 2008), and our results suggest that it also may be prevalent in vernal pool habitats. To our knowledge, this is the first documentation of such competitive genes in vernal pools.

4.6 Conclusions

These results suggest that resource addition, which greatly affected ecosystem function, may be accompanied by changes in bacterial community structure because the proportional representation of Acidobacteria and Alphaproteobacteria EGTs were higher with NO₃⁻ addition. However, we saw no alterations overall bacterial richness or
diversity with and without NO$_3^-$ addition, suggesting that processes which can be performed by many taxa, such as denitrification, are rather insulated from environmental changes. Thus, vernal pool microbial communities may have a high level of physiological plasticity, as evidenced by the metabolic diversity uncovered by the metagenomic approach. We also found a large number of sequences that matched with saprotrophic fungi and bacterial decomposers, as well as EGTs involved in organic matter degradation and a finding of EGTs suggesting competition between fungi and bacteria in vernal pools. This reaffirms previous conclusions that these habitats are heterotrophic with decomposition likely driving energy inputs and further suggests a high level of unexplored interactions between the organisms which comprise this detrital-based food web. As such, vernal pools may represent unique sites of microbial metabolic diversity and carbon and nutrient cycling in forest ecosystems.
Table 4.1 Sequence statistics for each vernal microcosm metagenome.

<table>
<thead>
<tr>
<th>Metagenome</th>
<th>MG-RAST ID #</th>
<th>Total Sequences</th>
<th>Matched&lt;sup&gt;a&lt;/sup&gt; Metabolic EGTs</th>
<th>Matched&lt;sup&gt;a&lt;/sup&gt; Phylogenetic EGTs</th>
<th>Unique&lt;sup&gt;b&lt;/sup&gt; Phylogenetic EGTs</th>
<th>Average Length (bp)</th>
<th>Longest Length (bp)</th>
<th>Shortest Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+NO₂⁻</td>
<td>4445106.3</td>
<td>28,437</td>
<td>5,252</td>
<td>9,733</td>
<td>592</td>
<td>317.57</td>
<td>555</td>
<td>16</td>
</tr>
<tr>
<td>--N</td>
<td>4445130.3</td>
<td>80,163</td>
<td>10,487</td>
<td>20,026</td>
<td>745</td>
<td>378.13</td>
<td>660</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>a</sup>The matched EGTs included only those that had an e-value of $10^{-5}$ or lower and had sequence similarity of 50 base pairs or greater.

<sup>b</sup>The unique EGTs were determined using the organismal level in MG-RAST. This represents species richness.
Table 4.2 Richness and diversity estimates of bacterial environmental gene tags from each metagenome using the organismal (i.e., species) level in MG-RAST.

<table>
<thead>
<tr>
<th>Metagenome</th>
<th>Richness</th>
<th>$H^a$</th>
<th>$D^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>+NO$_3$</td>
<td>486</td>
<td>3.931</td>
<td>0.9757</td>
</tr>
<tr>
<td>--N</td>
<td>594</td>
<td>3.969</td>
<td>0.9942</td>
</tr>
</tbody>
</table>

$^aH$ = Shannon-Wiener index of diversity  
$^bD$ = Simpson’s index of diversity
Table 4.3 Denitrification sequences from the SEED database that matched with environmental gene tags in each microcosm.

<table>
<thead>
<tr>
<th>Functional Role Assignment</th>
<th>SEED ID #</th>
<th>Alignment Length (bp)</th>
<th>e-value</th>
<th>% Identity</th>
<th>Metagenome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide reductase</td>
<td>fig</td>
<td>257309.1.peg .2143</td>
<td>103</td>
<td>1.00 x 10^-31</td>
<td>65</td>
</tr>
<tr>
<td>Nitric oxide reductase</td>
<td>fig</td>
<td>269799.3.peg .3461</td>
<td>72</td>
<td>6.31 x 10^-17</td>
<td>55</td>
</tr>
<tr>
<td>Nitric oxide reductase</td>
<td>fig</td>
<td>351745.7.peg .821</td>
<td>125</td>
<td>2.51 x 10^-43</td>
<td>64</td>
</tr>
<tr>
<td>Nitric oxide reductase</td>
<td>fig</td>
<td>269799.3.peg .3461</td>
<td>128</td>
<td>2.51 x 10^-43</td>
<td>57</td>
</tr>
<tr>
<td>Nitrous oxide reductase</td>
<td>fig</td>
<td>49338.1.peg.1792</td>
<td>123</td>
<td>2.51 x 10^-31</td>
<td>52</td>
</tr>
</tbody>
</table>
**Figure 4.1** The denitrification pathway. The reductases for each step are listed in the shaded boxes above each arrow and the genes for these enzymes are listed in the shaded boxes below each arrow.
Figure 4.2 Diagram outlining the methods used to analyze the metagenomes with the MG-RAST server.
**Figure 4.3** The proportional number of environmental gene tags from each metagenome that matched with “Nitrogen Metabolism” subsystem sequences in the SEED database at the second highest hierarchical level.
Figure 4.4 The proportional number of environmental gene tags from each metagenome that matched with subsystem sequences in the SEED database at the highest hierarchical level.
Figure 4.5 (previous page) The proportional number of environmental gene tags from each metagenome that matched with sequences in the SEED database in the a) “Carbohydrate” subsystem at the second highest hierarchical level and b) “Amino Acids” subsystem at the third highest hierarchical level. The graph of the “Amino Acids” subsystem includes only catabolic reactions.
Figure 4.6 The proportional number of environmental gene tags from each metagenome that matched with domain sequences in the SEED database.
Figure 4.7 (previous page) The proportional number of environmental gene tags from each metagenome that matched with a) bacterial and b) proteobacterial sequences of the SEED database after re-evaluation at the hierarchical levels 2 and 3, respectively, in the MG-RAST server.
Figure 4.8 The proportional number of environmental gene tags from each metagenome that matched with a) eukaryotic and b) fungal sequences of the SEED database after re-evaluation at the hierarchical levels 2 and 4, respectively, in the MG-RAST server.
Chapter 5

General Conclusions and Revision of Conceptual Model

The results presented here suggest that the relationship between microbial community structure and ecosystem function is highly context-dependent. Based on the conceptual model of Balser et al. (2001), I originally predicted the following (see Chapter 1 and Figure 1.1):

1. Fluctuations in pH should affect community structure of broad communities, such as bacteria and fungi, but not ecosystem processes due to high functional redundancy within these groups.

2. An addition of a resource (e.g., NO$_3^-$) should affect community structure of the functional group which utilizes that resource (e.g., denitrifiers, which use NO$_3^-$ as a terminal electron acceptor), as well as the process they perform, (e.g., denitrification).

3. Because pH can affect the solubility of chemical compounds, the interaction between pH and NO$_3^-$ addition should alter microbial community structure and ecosystem function beyond that of either alone.

Indeed, pH altered bacterial and fungal community structure and NO$_3^-$ addition markedly increased denitrification rate (Chapter 3). Contrary to the predictions, though, the altered bacterial and fungal community translated to differences in an ecosystem process (community respiration), while the increase in denitrification rate was independent of changes in microbial community structure, even of denitrifiers (Chapter 3) and even when bacterial diversity was profiled with a high throughput sequencing effort (Chapter 4). The conceptual model outlined in Chapter 1, then, needs to include the findings that modulator change can lead to alterations in widespread processes and that resource change can affect function independent of community changes, likely due to high metabolic diversity and physiological plasticity of functional groups (Figure 5.1).
However, resources, which have been defined in this dissertation as compounds used by functional groups as electron acceptors or donors, also serve as essential nutrients and their addition to a habitat can relieve nutrient limitation, especially in vernal pools, which are known to be oligotrophic (Carrino-Kyker and Swanson, 2007). In our microcosms, nutrient N addition increased denitrifier population size and community respiration (Chapter 3). Therefore, how resources affect microbial community structure and ecosystem processes depends on whether they are used by functional groups for respiration or used by many microorganisms as an essential nutrient. The conceptual model, then, should not only categorize environmental change as modulators or resources, but should further distinguish the effect of resources into respiration or nutrient affects (Figure 5.1).

The prediction that an interaction between pH and NO$_3^-$ would influence the relationship between community structure and function was also not upheld, as neither denitrifier community structure nor denitrification rate were affected by pH. An interaction between pH and N availability, however, did affect community respiration, again suggesting the importance of resources at eliminating nutrient availability. For the conceptual model, the interaction between modulator and resource may be realized only for ecosystem function if the resource is eliminating nutrient availability (Figure 5.1).

It is interesting that our metagenomic study was capable of detecting proportional gene changes for other aspects of nitrogen metabolism that were expected, but did not detect changes in denitrification genes. It is possible that our metagenomes contained genes from denitrifying organisms that have not been characterized, which seems likely since the microorganisms of vernal pools have been understudied (Colburn, 2004). The
identification of denitrification genes is an ongoing process; for example, denitrification was thought to be strictly prokaryotic until Shoun and Tanimoto (1990) found denitrifying activity in a fungus. Only the accumulation of genome sequences from newly isolated denitrifying organisms can increase sensitivity of the methods used to study these groups of organisms (Philippot and Hallin, 2006) and perhaps vernal pools harbor unique denitrifying organisms that have yet to be isolated. This is an interesting avenue for future studies on the biology of denitrifying organisms.

One aspect of vernal pools that has come out of this dissertation research is that they contain a high richness of saprotrophic fungi and bacterial decomposers as well as numerous genes involved in organic matter degradation (Carrino-Kyker and Swanson, 2008; Chapter 2; Chapter 4). Therefore, these habitats may be unique sites of nutrient cycling in forest ecosystems due to the presence of aquatic and aero-aquatic taxa that are not found in terrestrial soil (Bärlocher et al., 1978). Further, these organisms may be highly affected by changes in the nutrient quality of their carbon substrates. Even when investigating these communities at a relatively large landscape scale, local litter quality was highly related to differences in community structure (Chapter 2). Future studies should investigate how changes in nutrient quality and carbon availability affect vernal pool microbial community structure and function, including decomposition rate. Based on this dissertation research, though, such a study should consider that this relationship is context-dependent and that, though organic carbon availability may have a strong affect on organoheterotrophs, it likely will have no affect on lithoheterotrophs.

Finally, as microbial ecology continues to make advances on the relationship between microbial community structure and ecosystem function, it is imperative that
these studies not overlook the metabolic diversity of microbes. Because of this metabolic diversity, environmental changes that affect broad groups of microorganisms and widespread processes may not affect functional groups and the processes that they perform and different functional groups are likely differentially affected by resource fluctuations. Any change in these processes could affect microbial consortia, which comprise microbes of individual metabolisms that together are efficient at cycling nutrients in aquatic environments (Paerl and Pinckney, 1996). Therefore, by taking a context-dependent approach at studying the relationship between microbial community structure and ecosystem function, future studies are not limited to studying environmental effects on one functional group or one ecosystem process and, therefore, have the potential to advance our knowledge of how environmental change can affect microbial interactions and global nutrient cycling.
Figure 5.1 The revised conceptual model from this dissertation research. With a change in environmental pH, the realized microbial community and the functions they perform pass through two filters: a biochemical filter and a biogeochemical filter. The biochemical filter affects both community structure and ecosystem processes (represented by yellow arrows), while the impacts of the biogeochemical filter and the interaction between the filters, depend on whether the community is using chemical compounds as resources (arrows labeled “R”) or nutrients (arrows labeled “N”).
References


Stone, J.S. 1992. Vernal pools in Massachusetts: aerial photographic identification, biological and physiographic characteristics, and state certification criteria. MS thesis, University of Massachusetts, Amherst, MA.


