Spatial Patterns in Microbial Communities

Rima Bethany Franklin
Waiteville, West Virginia

B.A., Bard College, 1996

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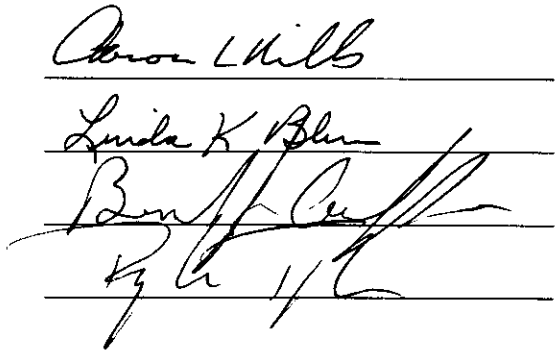

Chapter 1.

Introduction

1.1. Introduction

Because individuals can react only to their local environment, ecological interactions are intrinsically spatial. It is the local environmental that affects nutrient or food uptake, competition, or predation risk, and therefore indirectly controls growth, movement, reproduction, and survival. For microorganisms, the "local" environment is quite small, as an individual bacterium is usually less than 2 μm long. While some aspects of the environment (e.g., temperature and pressure) may be the same at the macro- and microscales, bulk measurements of other environmental variables (e.g., nutrient concentration and moisture content) may not accurately reflect the local conditions affecting an individual microorganism or a microbial assemblage. Nevertheless, most studies in microbial ecology are performed at larger spatial scales, using sample sizes that are determined by the researcher's perception of environmental variability or by the particular analytical technique to be employed, and rarely consider the small spatial scale at which individuals may actually be interacting with one another and the environment.

Brock (1987) proposed that in order to conduct appropriate microbial ecology, studies must focus on scales important to individual bacterial cells. He maintained that this is the "only way we can really see organisms in their actual environments," and "without knowing where these organisms lived, (how) can we make any sense" out of our ecological analyses. However, given that a single milliliter (1 cm^3) of unpolluted surface water typically contains ~ 10^6 bacteria, and soils can contain up to 10^{10} microorganisms in a single gram, it is a daunting task to study microbial ecology at the level of the

individual. Moreover, the small size of microorganisms and the hyperdiversity of microbial communities mean that Brock's challenge is currently insurmountable.

Not only are ecological approaches that rely on the identification and classification of individual members of a microbial community impractical, once completed, such a study would only be able to describe a very small geographic area within an ecosystem. However, the primary interest of most environmental scientists is in how microbial activity manifests at larger spatial scales and helps to control nutrient cycling, decomposition, primary productivity, and other microbially mediated ecosystem functions at scales that are relevant to humans. The total capacity of microbial communities at these larger scales can be thought of as the sum of the activity of several "unit communities" of microorganisms (Swift, 1984), in separate microhabitats, whose individual activities are pooled into what scientists observe at the field or landscape scale. In order to understand well how these units fit together and how their combined activity contributes to overall ecosystem function, we need to better understand the small-scale spatial distribution of microorganisms and microbial communities. In particular, we need to better understand the size and distribution of these unit communities (patches), the biological implications of the interactions among neighboring patches, and how variations in the macro-environment may alter these relationships and influence the activity of these patches.

Despite the importance of spatial variability in environmental microbiology, studies that specifically consider spatial scale when examining the distribution patterns of microorganisms are rare. Most often, when microbial ecologists publish papers describing the "spatial variation" or "spatial distribution" of bacteria in the environment,

they are either reporting the results of studies monitoring the distribution of individuals at the microscale (Dandurand et al., 1995; Dandurand et al., 1997; Dechesne et al., 2003; Grundmann and Debouzie, 2000; Jordan and Maier, 1999; Nunan et al., 2001), or they are discussing patterns observed at the landscape or regional scale (Blum et al., 2004; Cho and Tiedje, 2000; Finlay et al., 1996; Finlay et al., 1999; Finlay et al., 2001; Fulthorpe et al., 1998; Garcia-Pichel et al., 1996; Teske et al., 2000). There is much less research effort directed toward understanding spatial scale and variation in microbial communities at distances intermediate to those discussed above (i.e., from centimeters to hundreds of meters). In my dissertation research, I focused on these intermediate distances, and studied the distribution of microbial communities in several different environmental systems. Key questions addressed by this work include: (i) how are bacterial distributions structured and how do these patterns correlate with the distribution of environmental properties, and (ii) what are the relevant scales for sampling and understanding this spatial heterogeneity. Knowledge of the spatial patchiness of bacteria at these scales is important for addressing basic ecological questions, and has many consequences for experimental design, environmental sampling, and statistical analyses. Moreover, these distances are closest to what humans naturally perceive as they inspect an environment, so it is important to determine how observations made at these scales relate to microbial activity at other spatial scales. For example, scientists must understand the spatial relationship of microbial properties and resources at these (smaller) scales before they can be confident that their sampling designs are adequate to resolve differences at larger scales and in relation to ecosystem function and stability.

1.2. Background

1.2.1. Methodological limitations associated with community analysis

When macro-organismal ecologists set out to investigate community organization and spatial variability, the studies usually involve identifying the individuals in an area and recording their locations, relative to one another. However, there are a number of attributes of microbial communities that limit the use of such an approach, and many methodological constraints have thus far hampered our ability to study microbial diversity. In particular, the small size of microorganisms means that they are difficult to visualize. Even with the aid of a microscope, the lack of morphological distinctiveness among types makes the visual classification of individuals into different taxonomic groups impossible. Moreover, as discussed above, the tremendous abundance of organisms in microbial communities means that the task of sorting them is overwhelming. Another difficulty is in developing and implementing sampling methods that preserve the spatial distribution of microorganisms within the native environmental matrix during sample collection and processing. In addition, the hyperdiversity of microbial communities means that the use of such an individual-based approach is impractical in many cases; for example, microbial communities in soil have been shown to contain up to 10,000 types (species) in a single 30 g sample (Torsvik et al., 1996; Torsvik et al., 1998), and it has been proposed that the oceans may contain 2×10^6 types (Curtis et al., 2002).

Culture-based studies provide the framework from which microbial ecologists derive much of their current understanding of microbial interactions and community dynamics. However, it is well documented that cultural techniques are both selective and

unrepresentative of the total microbial community. Some studies propose that less than 1% of microorganisms in the environment can be cultured in the lab (Holben, 1997), though there is little solid evidence supporting the accuracy of this estimate. Since the application of molecular biological methods to microbial ecology in the mid-1980s, many new, previously uncultivated, microorganisms have been identified. Whole groups of organisms, known only from molecular sequences, are now believed to be quantitatively significant in many environments. In particular, the use of 16S rRNA gene sequences has brought about a new era of microbial systematics, and it has become quite popular to survey microbial community diversity using polymerase chain reaction (PCR) and 16S rRNA/DNA-based methods. The 16S rRNA genes contain highly conserved sequence domains interspersed with more variable regions, and comparative analysis of rRNA sequences can identify so-called "signature sequence motifs" that are targets for evolutionary-based identification (Theron and Cloete, 2000). The use of PCR amplification of 16S rRNA genes and subsequent cloning has allowed us to 'identify' a number of new 'species'; however, a tremendous portion of the microbial diversity has still not been explored. Current estimates indicate that between only 1 and 5% of the microorganisms on earth have even been identified and named (Kennedy and Gewin, 1997).

1.2.2. "Whole-community" approaches to microbial community analysis

In order to comprehend the full extent of the relationships within a microbial community, and between a community and its surroundings, researchers must be able to evaluate attributes for the assemblage without relying on microbial growth and culture-

based techniques for detection or identification of individuals. This need has lead to the development of several approaches that use "whole-community samples" for analysis of microbial communities. The basic premise behind this approach is that all of the organisms in a sample are analyzed as a unit, and relative comparisons are made between communities based on overall characteristics manifested by the different mixtures of organisms. In order to monitor structural differences in microbial communities, most of the research has focused on the analysis of whole-community DNA samples and several new molecular genetic approaches have recently emerged (to be discussed). Similarly, the lipid content of microbial cells (e.g., phospholipid ester-linked fatty acids or PLFA (Tulnid and White, 1990) and fatty acid methyl esters or FAME (Kennedy, 1995)) may be used to monitor community composition (Laczko et al., 1997; Zogg et al., 1997). However, many subsets of the microbial community respond to stressful conditions in their microenvironment by shifting lipid composition (Kieft et al., 1997; White et al., 1997), confounding the interpretation of the phospholipid patterns and signatures. Another commonly used whole-community approach is community-level physiological profiling (CLPP), where patterns in carbon substrate utilization are compared for different communities (Garland and Mills, 1991).

1.2.3. Molecular genetic techniques for comparing community structure

Since most of my dissertation research has used molecular genetic techniques to analyze microbial community structure, a brief overview of these approaches is warranted. For more detail on the use and development of these methods, see: Dahllof (2002), Johnsen et al. (2001), Kozdroj and van Elsas (2001), Theron and Cloete (2000),

and Torsvik and Ovreas (2002). As discussed above, most of the recent research using the whole-community approach in microbial ecology has focused on the analysis of the combined genetic material (either DNA or RNA) from a community sample. However, many of these techniques (e.g., DNA hybridization (Griffiths et al., 1996; Lee and Fuhrman, 1990; Lee and Fuhrman, 1991), percent G + C content (Holben and Harris, 1995), or DNA reassociation kinetics (C_0t curves) (Torsvik et al., 1990; Torsvik et al., 1994)) require a fairly large environmental sample in order to obtain enough genetic material for analysis. The need for large quantities of DNA often means that sample collection can be very time-consuming (e.g., filtering large volumes of water), and that samples may need to be gathered over a relatively large area (e.g., several grams of soil), making it impossible to examine small-scale spatial differences in community structure. Moreover, the analyses themselves are very time-consuming, which further limits the feasibility of large and comprehensive studies of microbial community dynamics. Technological development over the last several years has helped reduce this problem, and the introduction of PCR-based methods now permits more rapid analysis using smaller sample sizes.

Recently, the use of PCR-based "DNA fingerprinting" for the analysis of microbial communities has become very popular. Commonly used PCR-based DNA fingerprinting techniques include: denaturing gradient gel electrophoresis or DGGE (Muyzer, 1999; Muyzer et al., 1993), amplified ribosomal DNA restriction analysis or ARDRA (Massol-Deya et al., 1995), terminal restriction fragment length polymorphism or T-RFLP (Liu et al., 1997; Marsh, 1999), randomly amplified polymorphic DNA or

RAPD (Franklin et al., 1999; Wikström et al., 1999; Wikström et al., 2000; Williams et al., 1993), and amplified fragment length polymorphism or AFLP (Franklin et al., 2001; Franklin and Mills, 2003; Zabeau and Vos, 1993). These methods can be broadly categorized into two groups: (i) approaches where specific primers, designed to amplify certain known genes or sections of a genome, are used to direct the PCR (e.g., DGGE, ARDRA, and T-RFLP), or (ii) approaches where the PCR amplification is based on the distribution of random sequences throughout the DNA sample (e.g., RAPD and AFLP). When specific primers are used to study microbial communities, the 16S rRNA gene is most often considered.

There are several additional molecular biological techniques that have recently emerged for the study of microbial communities, and should be briefly mentioned. Specifically, the novel application of nucleic acid array technology to microbial community analysis may provide an efficient means to assess the presence of organisms or the expression of genes in communities. However, the performance of microarray hybridization in environmental studies has yet to be carefully evaluated, and a number of technological challenges need to be solved before this technique can reliable inventory complex samples (Zhou, 2003; Zhou and Thompson, 2002). Another important technique that is being refined is fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes in combination with microscopy or flow cytometry (Handelsman and Smalla, 2003). FISH has the unique potential to study the composition of bacterial communities *in situ* and may also be used to provide new ways to link structure and function in microbial ecology studies (Wagner et al., 2003). While these techniques

present a tremendous opportunity to examine microbial community dynamics in a wide variety of systems, they are nevertheless confined to ‘accessible’ and previously encountered bacteria. In order to apply either FISH or DNA microarrays, some portion of the genetic sequence of the individuals of interest must be available.

1.2.4. Spatial heterogeneity in microbial systems

In natural systems, environmental heterogeneity arises as a result of the interaction of a hierarchical series of interrelated variables that fluctuate at many different spatial and temporal scales. These physical, chemical, and biological variables may combine to influence the abundance, diversity, and activity of microorganisms at many different spatial scales. These properties do not vary independently; rather, the general perception is that any such variable measured at a certain point in space and time is the outcome of several processes, all of which are spatially variable. It is thought that the relative role of different environmental forces may vary across scales and among ecological systems, and one of the major challenges for the discipline of ecology is to measure the relative strengths of these factors in natural ecosystems, examine the interactions among them, and combine this information in an effort to explain the patterns of organism distribution, abundance, and function.

Studies of spatial organization in microbial systems may be broadly categorized into four scales of interest: microscale, plot scale, field or landscape scale, and regional scale (Parkin, 1993). Within each of these scales/categories, multiple levels of organization may exist. Often, the hierarchical levels are nested so that high-level units consist of aggregations of lower-level units, though the boundaries between levels are not

usually visible. Many of the studies that have considered spatial variability in microbial ecology focus on a single scale, though it has been suggested that, because of the hierarchical nature of spatial variability, multi-scale analyses of spatial variability are needed in order to fully represent the complexity of natural systems (Benedetti-Cecchi, 2001).

Because spatial variability can manifest at many different scales, the patterns one observes depend greatly on the scale of observation (Avois et al., 2000; Levin, 1992). In sampling theory, spatial scale is defined by several characteristic properties: grain size, sampling interval, and extent (Legendre and Legendre, 1998). *Grain size* is the size of the elementary sampling units (e.g., the volume of sample), and defines the resolution of the study (Schneider, 1994). *Sampling interval* is the average distance between sampling units, and the *extent* is the total area included in the study. Depending on the ecological question being addressed, and what is already known about the scale of the process of interest, the dimensions of these components vary. For a given sampling design, no structure can be detected that is smaller than the grain size or larger than the extent of the study. In this way, the sampling design defines the observational window for spatial pattern analysis (Legendre and Legendre, 1998).

1.2.4.1. "Local" controls on the spatial distribution of microorganisms

Though the primary focus of my dissertation research has been to study the spatial distribution of microbial communities at larger spatial scales (cm to plot-scale), it is important to understand the factors that control the distribution of individuals and populations at the microscale in order to determine which variables may be useful for

study at larger scales. Microorganisms are generally regarded as inhabiting "microhabitats", but this term is poorly defined and the meaning differs for different types of organisms (e.g., fungi versus bacteria) and in different systems (e.g., soil versus aquatic). For example, bacterial development in soils is probably influenced by conditions within only a few microns, while a fungus has the advantage of being able to extend beyond its initial immediate surroundings, using its hyphae in much the same way that a plant root system does (Harris, 1994). For this reason, a fungus may experience a degree of averaging of soil conditions, and is not restricted to as small of a microhabitat as a bacterium (Parkin, 1993). In aquatic systems, the more diffuse nature of the environmental matrix may mean that microbes are impacted by environmental variability existing at a broader spatial scale, compared to a more highly structured soil matrix. The size of a microhabitat may be defined by the physical and chemical environment directly adjacent to the microbial cell or colony (Parkin, 1993), and, in this regard, is not a fixed unit. Its size is operationally dependent upon the specific process or microorganism under study, and the nature of the environmental matrix within which the organism resides.

The distance between and "reachability" of different microhabitats is an important issue that may help control the spatial distribution of microorganisms and microbial community composition. For an organism to be present in a system, it must either evolve there or be transported from another site, so the spatial continuity of microhabitats may help control the distribution patterns of microorganisms at many different scales. Moreover, spatial continuity and transport of microorganisms may influence the response of a microbial community to a disturbance. In particular, the frequency or extent to

which a disturbed system is inoculated with new organisms from “nearby” or “connected” communities could have a strong influence on system recovery. Similarly, this type of information could be useful for predicting the distribution and persistence of a non-native or invasive microorganism in an ecosystem. Practical applications include determining the distribution of plant pathogens in an agricultural system or judging the success of an intentionally introduced organism placed in a contaminated environment for the purposes of bioremediation. At this point, it is unclear what the relationship is between spatial heterogeneity and colonization success for these types of organisms. Colonization success may be greater in a heterogeneous system, because a spatially heterogeneous environment is more likely to include a microenvironment that is hospitable to the new organism. However, in a diverse and spatially heterogeneous habitat, the number of occurrences of this ideal microenvironment may be small, in which case spatial heterogeneity may make it more difficult for an invasive/introduced organism to achieve dominance and thus have a major influence on the ecosystem**.**

Bacterial colonization can occur due to active movement of an organism to a new site, or through passive transport by other agents (e.g., water or animals) (Harris, 1994). Though little is known about the importance of bacterial motility on colonization, it is generally assumed that active movement is relatively small compared to other dispersive processes. More research is necessary to investigate the relative importance of these two transport pathways, and the spatial extend over which each may be important. A central question that follows from an investigation of transport of microorganisms is to what extent can an isolated cell survive and successfully colonize a given location. After a cell arrives at a colonization site, it could exist in a resting stage for some period of time, it

could die, or it could grow and reproduce, potentially providing a seed for further colonization of another location. Which of these scenarios takes place is likely determined by resource availability and by interactions with other community members. The ability to predict colonization efficiency then requires increased research into several questions of fundamental ecological importance, including habitat suitability, invasibility of existing communities, and interactions among community members (e.g., competition, predation, and synergistic or mutualistic relationships). As scientists learn more about these phenomena, especially at scales relevant to individual microbes, we will become better able to predict the persistence of unique organisms in a new habitat. This type of knowledge about the microscale variation in microbial communities is necessary for understanding the mechanisms behind microbial community formation and maintenance, and for evaluating the stability and resilience of these communities.

In addition to the topics discussed above, spatial heterogeneity may help control community composition and diversity by altering biological interactions among organisms and through habitat partitioning. In particular, it is thought that spatial heterogeneity plays an important role in determining diversity, as spatial structure in microenvironments can increase niche complexity. This increased niche complexity may create favorable habitat space for many types of organisms, with very different physiological requirements, within a rather small area. Similarly, if the habitat is subdivided into many separate pockets of resources, populations may avoid competition by physical isolation, and this is thought to contribute to the tremendous microbial diversity seen in soils (Zhou et al., 2002). Habitat partitioning can also influence predation, and thus exert a strong indirect control on community composition.

The plausibility of spatial structure (e.g., patchiness) at small scales in microbial systems has been intensively debated in the past (Azam and Ammerman, 1984; Fenchel, 1984; Lehman and Scavia, 1982; Levin and Segal, 1976; Sieburth, 1984); however, a great deal of evidence is now available to demonstrate that this type of microscale patchiness is widespread (Blackburn and Fenchel, 1999; Blackburn et al., 1998; Duarte and Vaqué, 1992; Grundmann and Debouzie, 2000; Krembs et al., 1998a; Long and Azam, 2001; Nunan et al., 2003). A prerequisite for such an analysis is the conservation of the native state of the environmental sample such that the *in situ* distribution of the inhabitants and the environmental components are preserved. One strategy for investigating microorganisms within their natural spatial distribution is by embedding the samples in a material such as agarose (Macnaughton et al., 1996), paraffin wax (Licht et al., 1996; Poulsen et al., 1994; Rothemund et al., 1996), and hard setting resins (Kawaguchi and Decho, 2002; Manz et al., 2000; Nunan et al., 2001) prior to analysis. In aquatic systems, a spatial information preservation (SIP) method has been applied, which is based on rapidly freezing small samples of water as a means of maintaining the 3D particle distribution for microscopic analysis (Krembs et al., 1998a; Krembs et al., 1998b).

In soils, one-dimensional microscale data has been collected along soil transects (Grundmann and Debouzie, 2000) and along plant roots (Dandurand et al., 1995), and non-random spatial patterns of bacteria have been identified. More recently, efforts have focused on analyzing the two- (Dandurand et al., 1997; Nunan et al., 2001) and three-dimensional (Dechesne et al., 2003; Grundmann et al., 2001) distribution of microorganisms by integrating the analysis of multiple “microsamples.” The results

indicate that the microhabitat distributions in soil probably involve an array of colonized patch sizes, and the location of different nutrient sources is thought to be one of the major factors determining the distribution of bacteria in soil (Dechesne et al., 2003). For example, the distribution of particulate carbon may have a strong influence on the small-scale variations in bacteria abundance (Parkin et al., 1987; Wachinger et al., 2000). However, the situation is more complex, and less well understood, for soluble substrates (Dechesne et al., 2003). In aquatic systems, most of the previous work on very small-scale patchiness has been based on "cluster" hypotheses, including the proposal that bacteria actively congregate around phytoplankton cells (i.e., the phycosphere concept (Azam and Ammerman, 1984; Bell and Mitchell, 1972)) or particulate organic matter (Long and Azam, 2001) to enhance their exposure to growth substrates. For example, direct manipulation of water samples via the addition of algal detritus has been shown to stimulate the formation of nano-scale patches of lake bacterioplankton (Krembs et al., 1998a). In order to determine the biological and environmental significance of this type of patchiness, it will be necessary to determine how common the phenomenon is in space and time and in different environments. If patchiness at these scales is widespread, as many researchers now believe, it may mean that rate processes that are concentration dependant are being miscalculated (Krembs et al., 1998a).

1.2.4.2. Variability at larger spatial scales

In general, the grain size used for collecting environmental samples of microbial communities is too large to permit analysis of the location or activity of individual organisms, and most of the work looking at microbial community spatial variability

examines larger scales. Studies in agricultural soils have demonstrated that significant spatial heterogeneity may exist for microbiological processes (Bending et al., 2001; Grundmann and Debouzie, 2000), community structure (Balser and Firestone, 1996; Cavigelli et al., 1995; Franklin and Mills, 2001), and abundance (Nunan et al., 2001; Wollum and Cassel, 1984); patch size estimates range widely from as small as 2 mm (Grundmann and Debouzie, 2000) to nearly 10 meters (Franklin and Mills, 2001). Similar studies have been conducted in grassland and forest soils (Both et al., 1992; Kuperman et al., 1998; Morris, 1999; Ritz et al., 2001; Robertson et al., 1988; Saetre and Bååth, 2000), in a shallow coastal aquifer (Franklin et al., 1999), and in the open ocean (Duarte and Vaqué, 1992; Mackas, 1984). For salt marsh and marine sediments, variation has been examined at small scales (i.e., < 1 m^2 (Berardesco et al., 1998; Danovaro et al., 2001; Franklin et al., 2002; Scala and Kerkhof, 2000)), and at intermediate (< 150 m (Moran et al., 1987; Scala and Kerkhof, 2000)) and larger distances (km (Scala and Kerkhof, 2000)). In general, all of these studies reveal that microbial communities can be organized at a variety of spatial scales, which likely reflect the scales of heterogeneity in the distribution of physical and chemical properties for the environment under investigation. Most of this work has considered more general community properties (e.g., total abundance, biomass, or activity (Duarte and Vaqué, 1992; Moran et al., 1987; Morris, 1999)), while relatively few studies have examined the distribution of microbial community structure (Balser and Firestone, 1996; Both et al., 1992; Mackas, 1984; Saetre and Bååth, 2000).

1.2.4.3. Hierarchical scales of organization

Given that environmental factors do not necessarily operate independently, or at distinct spatial scales, studying microbial systems using a single analytical scale cannot provide a complete understanding of community dynamics. Multi-scale comparisons, in which patterns are analyzed at several different spatial scales, may be more useful when trying to identify the factors that control community development. Conclusions about the organization of microbial communities, the effect of disturbance, or the roles of various limiting factors are likely to differ at different spatial scales (Wiens et al., 1986). Moreover, the characterization of microbial communities at several different scales may help explain paradoxes that arise when different investigators, studying similar communities but at different scales, arrive at different conclusions about the factors that structure those communities. These disagreements may reflect viewpoints of different scales, and not differences in the way communities are organized (Rahel, 1990).

Recently, scientists have begun to focus on multi-scale comparisons, and have found evidence for nested scales of spatial structure in microbial communities (Ettema and Wardle, 2002; Robertson and Gross, 1994; Saetre and Bååth, 2000; Stenger et al., 2002). For example, Nunan et al. (2002) studied the spatial distribution of soil bacteria at three different scales, ranging from μm to meters, and found that the distribution of individual bacterial cells was organized at two scales in the subsoil, and at a single scale in the topsoil. Studies conducted in agricultural and shrub-steppe ecosystems suggest that microbial biomass and activity may be spatially dependent at scales less than 1 m, nested within a larger scale related to variations at the landscape level (Robertson et al., 1997; Ronimus et al., 1997; Smith et al., 1994). The presence of nested scales of

variation suggests that the various factors regulating the development of microbial communities in the soil ecosystems may operate at different scales (Robertson and Gross, 1994), and a simultaneous analysis of the multi-scale spatial variability of microbial community structure and the associated microenvironment could help identify these factors and determine their relative influence.

1.3. Research Motivation

Increased research into the spatial distribution of microorganisms and microbial communities has many ecological and environmental applications. For example, scientists are often interested in understanding issues of scale (spatial and temporal), in part, because of a desire to make predictions about ecosystem processes using information gathered at a smaller scale, or vice versa, i.e., upscaling and downscaling (Stein et al., 2001). This is a pressing issue because calculations of the effects of human activities on ecosystems often need to be made at spatial scales that far exceed the scale at which measurements are made (Schneider, 1994). For example, rates of nutrient processing through an ecosystem are generally calculated based on information measured for a few sampling locations. However, a direct scale-up of these rates is not appropriate unless one assumes that the factors that influence nutrient cycling are distributed homogenously across the landscape and over time. The reliability of such an estimate may be greatly increased by incorporating some information about the spatial and temporal distribution of the process of interest into any models or calculations (Schneider, 1998). Moreover, the increase in spatial scale may result in new interactions

and relationships, and a change in system organization, so that a change in the level at which one wishes to understand or quantify a process cannot necessarily be addressed by simply changing scale (O'Neill and King, 1998).

Another important issue for scientists designing and planning field experiments is resolution, and the need to make decisions about the appropriate scale for collecting data. In some cases, information collected at finer scales may be too noisy, and may obscure the detection of large-scale relationships. Alternately, our ability to detect relationships between large-scale processes may be inhibited by the loss of fine-scale information; for example, Hewitt et al. (1998) detected fewer relationships between environmental variables and communities using coarser resolution in a comparative study. Ultimately, the scale used for an analysis must be determined based on the processes of interest, and different scales may be appropriate for different ecological questions. This information is necessary for scientists to design effective sampling schemes for the environment, and changing the number, location, and size of samples collected may influence one's results. For instance, Parkin et al. (1987) studied the effect of sample size on determination of soil denitrification rates, and found that smaller samples provided significantly lower estimates of the mean denitrification rate than did larger samples.

While many ecological theories and models acknowledge that elements that are close to one another in space or time are more likely to be influenced by the same generating processes, the same energy inputs, or a similar physical environment (Legendre and Fortin, 1989), the classical statistical procedures employed to analyze these phenomena assume independence of observations. Statisticians generally count one degree of freedom for each independent observation, which allows them to choose an

appropriate statistical distribution for testing; the lack of independence that arises from the presence of spatial autocorrelation makes it difficult (in many cases, impossible) to accurately determine the number of degrees of freedom and correctly perform tests such as correlation, regression, or analysis of variance. Positive autocorrelation reduces within-group variability, artificially increasing the amount of among-group variance, and often leads to the determination that differences among groups are significant, when in fact they are not (Legendre et al., 1990). Violations of the assumption of independence and inappropriate application of these statistical procedures to spatially autocorrelated data may lead to incorrect conclusions. Therefore, understanding the type and extent of spatial variation in microbial systems is necessary in order to perform appropriate statistical analyses and to design reliable sampling schemes for the environment.

Increased knowledge of the spatial distribution of microorganisms and microbial communities in the environment also has many environmental applications, e.g., determining the impact of various land management practices on microbial communities or estimating biodegradation rates. In particular, agricultural land management practices have been shown to reduce heterogeneity in soil characteristics, which may influence the microbial community and nutrient cycling. Webster et al. (2002) found a decrease in the diversity of certain microbial populations in response to a fertilizer application, and Parry et al. (1999) correlated differences in denitrification rates between pasture and cropped soil with differences in pore space structure in soil clods. Biodegradation rates may also be strongly influenced strongly by the spatial heterogeneity of environmental conditions and microbial distributions at many different scales. At the microscale, the placement of certain organisms, relative to transport pathways through the soil matrix or substrate

availability, could be particularly important. For example, the spatial distribution of bacteria in soil, relative to pore networks or organic matter deposits, is thought to influence the degradation of groundwater pollutants (Nunan et al., 2001). At larger scales, high spatial variability is a key problem when quantifying methane emissions from soils at both the meter scale (Adrian et al., 1994; Wachinger et al., 2000) and the landscape scale (Valentine et al., 1994), and it is thought that different processes are responsible for this variation at different scales. Wachinger et al. (2000) demonstrated that CH_4 production was strongly correlated to the presence of fresh organic carbon at the cm- to meter-scale, while hydrologic regime was important at larger scales.

1.4. Research approach

For my dissertation, I examined the local (relatively small-scale) spatial distribution of microbial community structure in the environment, considering a number of different ecological settings and a variety of different spatial scales (from nl to ml, and from centimeters to hundreds of meters). These issues were addressed through a series of field studies and laboratory microcosm experiments designed to consider many issues related to spatial variability, including the importance of grain size, sampling extent, and environmental heterogeneity. Overall, this document presents empirical evidence and theoretical arguments that demonstrate the importance of a spatially explicit approach to the study of microbial communities, and it specifically addresses the implications of spatial variability for the structure and function of microbial communities. General issues addressed include: (i) How are microbial communities organized in space? (ii) Can these distribution patterns be explained by considering spatial heterogeneity in the

physical-chemical environment? What does this tell us about the environmental factors that are important for controlling microbial community structure? and (iii) How should spatial variability be considered in experimental sampling designs and statistical analyses?

Analyzing the distribution of community structure can provide some information about the scale at which microbial communities organize in the environment and may indicate relevant scales for sampling and studying microbial community structure. However, without a good understanding of how community structure and function are related, this information is of somewhat limited value for researchers who also want to understand the distribution of microbial community activity in the environment. Understanding the relationship between the structure and function of a community is necessary before scientists can anticipate how habitat disruption may influence the performance of a microbial community in an ecosystem, and is especially important if one wishes to use structural assays to make these predictions. To this end, a second portion of my dissertation research has focused on comparing community structure and diversity with community function and examining the role of functional redundancy in microbial systems. For these experiments, I used laboratory batch culture experiments to examine how manipulation of overall diversity (via serial dilution) can impact the *in situ* function and overall functional potential of a microbial consortium. Because the dilution procedure is essentially obtaining smaller and smaller samples from the environment, these studies can also be interpreted as looking at how microbial community structure and function change at very small scales (from 1 nl to 1 ml total sample size).

1.5. Dissertation organization

The chapters of my dissertation take the form of several manuscripts. Most of these manuscripts have already been completed and published, and I expect to submit the final two shortly. These chapters generally follow the format of a standard scientific article, and each includes an independent abstract. A single reference list has been compiled, and is included at this end of this dissertation. Given that each research paper was originally written to stand alone, some of the introductory material in each chapter is repetitive. This is particularly true when different investigations used common methodologies. I have chosen to leave these sections unmodified in order to preserve the integrity and consistency of the original research papers. The reader may notice that, in some situations, the statistical or analytical techniques presented in later chapters could also have been applied to datasets from the earlier experiments. Rather than presenting a reanalysis of these earlier experiments, only the original work is provided. However, any situations where the statistical methods developed in later chapters would have influenced the conclusions of the earlier research have been noted and discussed.

1.6. Summary of specific research objectives

The research objectives of this dissertation may be divided into three general categories: (i) development of analytical methods for microbial community analysis, (ii) analysis of spatial structure in microbial systems, including issues related to statistical techniques and experimental design, and (iii) questions regarding the relationship of

microbial community structure and function. The research conducted to address these questions may be conveniently divided two groups: laboratory and field experiments, and specific objectives for each category are presented below.

1.6.1. Laboratory experiments

In order to investigate the role of sample size on the analysis of microbial community structure and function at very small spatial scales, a series of microcosm experiments were performed using serial dilutions of a sewage microbial community to inoculate a set of batch culture experiments in sterile sewage. Regrowth of the diluted mixture was used as a way to generate sufficient biomass to analyze, and each regrown culture represented the composition of progressively smaller fractions of the original community. These experiments provided a means of comparing community structure and function at very small scales, as samples corresponded to a habitat size of 1nl to 1 ml (community size: ~ 1 cell to 10^8 cells). This approach allowed us to consider differences over several orders of magnitude, which could not have been accomplished practically with larger sample sizes (e.g., 1 ml, 100 ml, 1 L, 10 L, 100 L, etc.). A main objective of this work was to determine how the structure and function of communities in smaller spatial units differs from that determined by measuring larger spatial units.

These experiments also addressed the issue of functional redundancy in microbial communities and provided a way of studying how microbial community function may change as community structure changes. In addition to differences in effective sample size of the microbial community being considered, the dilution/regrowth technique provided a way of systematically manipulating microbial community diversity.

Probability suggests that dilution of a relatively diverse community will remove rare organism types, creating mixtures of cells differing in species richness in each successive dilution. Regrowth of these diluted mixtures should then produce cultures of roughly the same biomass, but differing in overall diversity. The objectives of this portion of the dissertation were:

- ***To examine the relationship between sample size and microbial community analysis.*** In particular, these studies considered how small a "community" sample can be and still have the same structure and functional ability as a larger sample.
- ***To compare several procedures for monitoring microbial community structure*** and to determine whether genotypic assays (i.e., DNA-based techniques) provide results similar to phenotypic ones (e.g., CLPP).
- ***To examine the relationship of microbial community structure and diversity to community function.*** The importance of functional redundancy in diverse microbial communities was studied by examining how the removal of organism types from the mixture impacted the overall *in situ* function of the community.

Chapters 3 and 4 of this dissertation present the results of the dilution/regrowth experiments. In general, the discussion focuses on the interpretation of these results as they relate to the 2nd and 3rd objectives listed above, as the implications of the work in that area are less straightforward than the interpretation with regards to the issue of sample size. Chapter 3 focuses on the development of the dilution/regrowth approach, and compares the results of several microbial community analyses with a series of numerical simulations, while Chapter 4 examines the relationship between microbial community structure, diversity, and community function.

Part of the goal of these lab experiments was to compare several different procedures for monitoring microbial community structure. In addition to the comparisons presented in Chapters 3 and 4, I also performed some research to adapt two DNA fingerprinting techniques (RAPD and AFLP), commonly used in population biology, to the analysis of microbial communities. Chapter 2 discusses the application of RAPD to the analysis of microbial community structure in stream and groundwater samples, and includes a discussion of the relationship of these types of DNA fingerprinting techniques with other whole-community assays. The application of AFLP to microbial community samples is presented as part of Chapter 3.

The experimental results presented in Chapters 3 and 4 were in good agreement with the numerical simulations (Chapter 3), and demonstrate that the dilution/regrowth approach is a useful means of creating communities varying in overall diversity and community structure. Moreover, the findings from the various analytical techniques were quite similar, and the genotypic and phenotypic assays were consistent with one another. However, different methods were sensitive to different types of changes in community structure (e.g., richness versus evenness), and these results highlight the importance of using multiple approaches to compare microbial communities.

Chapter 4 specifically addresses the issue of functional redundancy in diverse microbial communities, and uses the dilution/regrowth approach to examine the relationship between microbial community structure, diversity, and *in situ* function of a sewage microbial community. Heterotrophic uptake experiments were performed using five different ^{14}C labeled substrates, and there were no significant differences between communities in either the rate of uptake of a substrate or the assimilation efficiency for

any of the compounds studied. The fact that function was maintained, despite the loss of diversity and change in community structure, indicates that functional redundancy was quite high within the original microbial consortium and may have conferred some functional stability on the assemblage. For each organism type eliminated during the dilution process, at least one of the remaining organism types was able to provide the same function at the same level as the lost type.

With regards to sample size and spatial issues, the results presented in Chapters 3 and 4 indicate that different patterns of community structure may be detected by collecting samples of different sizes, but this relationship is controlled by the relative distribution of the different organism types. If the community has a relatively even distribution (approximately equal numbers of each type), then changes in sample size will not necessarily lead to changes in the perceived community structure – until the sample size is so small that the number of organisms collected is less than the richness present in the largest sample size. This result was not particularly surprising, and implies that differences in community structure will be only detected when samples sizes differ by several orders of magnitude. However, the results indicate that very large differences in community structure may be observed over relatively small intervals (e.g., 0.1 ml to 1 ml) if the community is not evenly distributed.

1.6.2. Field studies

For this portion of my dissertation, I chose three sites on Virginia's Eastern shore: a shallow groundwater aquifer, salt-marsh creek bank sediment, and an agricultural wheat field. The microbial communities inhabiting these areas were sampled at a variety of

spatial scales (from 2.5 cm to more than 150 meters), and compared using geostatistical techniques. Whenever possible, information on the physical-chemical environment associated with each sample was also collected to determine if habitat variability was correlated with differences in microbial community structure.

At two of the sampling sites (the groundwater aquifer and the salt-marsh sediments), the environmental heterogeneity was such that sets of samples were taken from different chemical or hydrological zones, but were spatially proximal and environmentally contiguous. For these sites, I considered the spatial distribution of communities within and across zones. The wheat field site was selected because of its *relative* homogeneity of environment (at the scale the researcher could perceive) in hopes that I would be able to study the spatial distribution of the communities with fewer confounding factors than in the first two studies. In addition, a sampling scheme was developed that allowed me to assay for multiple scales of organization within a single plot. The specific objectives of this portion of the dissertation were:

- ***To demonstrate the presence of spatial autocorrelation in microbial community structure*** in heterogeneous and homogeneous (at the scale perceived by the researcher) environments.
- ***To quantify the microbial community patch size in each environment***, and to estimate the spatial dependence (the percent of variance in that data that can be explained by considering the spatial separation of the sampling units) in each system.
- ***To investigate the presence of multiple scales of spatial organization in microbial systems.***

- ***To compare the correlation length scale of the microbial communities with the correlation length scale of the physical-chemical habitat variation,*** in order to evaluate whether spatial patterns in microbial properties can be linked to chemical or physical factors that affect, or are affected by, microbes directly.

- ***To determine whether larger-scale differences in community structure may be correlated with overall differences in the physical-chemical habitat.***

- ***To examine whether different microbial properties (e.g., abundance and community structure) have different distribution patterns.***

Chapter 5 presents the results of the study performed at the shallow groundwater aquifer, which contained distinct regions of anaerobic and aerobic groundwater. Several wells in each region were sampled, with separation distances ranging from 10 m to more than 150 m, and RAPD fingerprinting was used to analyze microbial community structure. Within the aerobic zone of the aquifer, where the groundwater chemistry was fairly homogeneous, the communities from the various wells were quite similar; the degree of similarity among the communities in the anaerobic region was less, and reflected a higher level of variation in the chemical conditions in that portion of the aquifer. There was no relationship between the genetic relatedness of a pair of communities and their spatial separation, either within or between chemical zones, suggesting that community patch size was smaller than the smallest sampling separation distance (10 meters). However, a strong correlation between environmental similarity and community similarity was detected, and the results indicate that the communities may track spatial and temporal variation in the environment to the extent that they may converge genetically as their environments become more similar.

Chapter 6 presents the results of a similar study performed to characterize the fine-scale spatial variations of microbial communities in sediment obtained from a salt-marsh creek bank. Samples (1 cm^3) were collected at 5-cm intervals along a 215-cm horizontal transect, over a 50-cm elevation gradient. A geostatistical analysis demonstrated a strong spatial autocorrelation for both bacterial abundance and community structure (RAPD DNA fingerprinting). The results indicate that processes more correlated with elevation vary at a smaller scale (therefore producing smaller patch sizes) than processes controlled by distance from the creek bank. Moreover, the processes that control microbial abundance in this system operate at a scale larger than those that control community structure. As in the groundwater study, a strong coupling of physical-chemical environmental and microbial community structure was observed. In particular, the study suggests that factors such as inundation frequency, during and extent of flooding, sediment moisture content, and sediment redox status may be important factors controlling microbial community structure.

To compare with the earlier studies examining the spatial distribution of microbial communities in heterogeneous environments, I also examined the spatial variability of microbial communities in an agricultural wheat field. Nearly 200 soil samples were collected at a variety of separation distances ranging from 2.5 cm to 11 meters. The analysis of microbial community structure in this field is presented in Chapter 7. In general, the results indicate a remarkable degree of spatial structure was present in this pedagogically homogeneous site that has been ploughed and cropped as a single field for several years. Multiple scales of spatial autocorrelation were found, and, in some

locations, up to four distinct correlation length scales were detected. Maps of the spatial distribution of community structure indicate that different portions (subsets) of the microbial community had different distribution patterns.

In an effort to better understand the factors that may influence microbial community organization at various spatial scales in soils, the results presented in Chapter 7 were compared with an analysis of the distribution patterns associated with several soil physical-chemical properties (soil carbon (C), nitrogen (N), organic matter (OM), and texture (sand, silt, and clay content)). Geostatistics demonstrated the presence of multiple scales of spatial autocorrelation for all of the environmental variables, and the patterns on the kriged maps were similar to those previously observed for microbial community structure. Simple causal modeling was used to study the direct relationship between each environmental property and each microbial community property, and the results indicate that there is a strong correlation between these two sets of variables, in excess of their shared spatial patterns. In general, soil C and N content was strongly correlated with community structure at all of the scales considered, while other properties (OM and texture) were only correlated with specific subsets of the community. The models developed for larger spatial scales were more complex and indicated a stronger role of spatial heterogeneity in controlling microbial community structure, compared to models developed for finer spatial scales ($\leq$ 40 cm).

Chapter 2.

Characterization of Microbial Communities Using Randomly Amplified Polymorphic DNA (RAPD).

Franklin, R. B., D. R. Taylor, and A. L. Mills. 1999.
Journal of Microbiological Methods. 35:225-235.

Abstract

Similarity among a number of aquatic microbial communities was examined using Randomly Amplified Polymorphic DNA (RAPD), a common polymerase chain reaction (PCR)-based DNA fingerprinting technique. After amplification of whole-community DNA extracts, the PCR products were resolved by agarose gel electrophoresis and the band patterns compared to determine percent similarity. Twelve different primers were used to amplify approximately 100 fragments (total) from each DNA sample; the bands were scored as present or absent and the similarity between each sample was determined using Jaccard's coefficient. From this information, dendrograms were constructed and a bootstrapping procedure was used to assess how well supported the tree topologies were. Principal component analyses (of the presence/absence data) were also conducted as a means of visualizing the relationships among samples. Results obtained for two different experimental systems (a pair of tidal creeks and several wells in a shallow groundwater aquifer) correlated well with the temporal and spatial variations in environmental regime at this sites, confirming that arbitrarily primed PCR-based DNA fingerprinting techniques such as RAPD are useful means of discriminating among microbial communities and estimating community relatedness. Moreover, this approach has several advantages over other DNA-based procedures for whole-community analysis; it is less laborious and uses smaller quantities of DNA, making it amenable to sample-intensive monitoring, and it does not depend on culturing or the use of selective PCR primers.

2.1. Introduction

The abundance and diversity of microorganisms in a given environment is typically enormous. As a result, it is not possible to get a complete sense of the relative numbers and identities of the constituent organisms in a microbial community with any of the currently available analytical techniques. It is similarly impossible to thoroughly understand the function of each individual organism type, the specific interactions that may exist between populations, or the independent influences organisms may have on ecosystem processes. This inability to completely categorize the constituents of a community has hampered the efforts of microbial ecologists to investigate fundamental ecological concepts such as community diversity, succession, redundancy, or stability.

Culture-based studies provide the framework from which microbial ecologists derive much of their current understanding of microbial interactions and community dynamics; however, the fraction of organisms that have been cultured is thought to be less than 1% of the total (Holben, 1997). In order to comprehend the full extend of the relationships with a microbial community, and between a community and its surroundings, researchers must be able to evaluate attributes for an entire community without relying upon microbial growth for detection. This need has led to development of several approaches that use whole-community samples for analysis, with much of the research focusing on the use of whole-community DNA extracts (Griffiths et al., 1996; Holben, 1997; Ogram and Feng, 1997). Commonly used techniques include DNA hybridization (Griffiths et al., 1996; Lee and Fuhrman, 1990; Lee and Fuhrman, 1991), percent G+C content (Holben and Harris, 1995), DNA reassociation (Britten et al., 1974;

Torsvik et al., 1994), and RFLP (Restriction Fragment Length Polymorphism (Stahl, 1997)). The primarily limitation of these procedures is that they require rather large amounts of relatively pure DNA for analysis. The need for large quantities of DNA often means that sample collection can be very time consuming (e.g., filtering large volumes of water), and that samples may need to be gathered over a relatively large area (e.g., several grams of soil or sediment), thus making it impossible to examine small-scale spatial differences in community structure. Moreover, the analyses themselves are very time consuming, further limiting the feasibility of large and comprehensive studies of microbial communities.

The application of polymerase chain reaction (PCR) to microbial ecology has helped to reduce some of these problems, in particular those resulting from the need for large quantities of DNA, and has eliminated the need for extensive concentration of cells from environmental samples. PCR has proven especially useful in the study and comparison of the DNA sequences of the genes coding for rRNA. Some of the rDNA studies compare individual sequences to a database of previously encountered (generally cultured) organisms (Devereux and Willis, 1995) in order monitor the distribution of "phylotypes" (distinct groups of related DNA sequences) in the environment (Wise et al., 1997). Inevitably, the comparison, and the database construction, includes some cultural bias. Other researchers studying rDNA have taken a different approach – without attempting to link the sequences with known taxa, they examine the diversity of "fingerprints" derived from manipulation of rDNA sequences (e.g., ARDRA – Amplified Ribosomal DNA Restriction Analysis (Massol-Deya et al., 1995; Weidner et al., 1996),

DGGE – Denaturing gradient Gel Electrophoresis (Ferris et al., 1996; Øvreås et al., 1997), and T-RFLP – Terminal Restriction Fragment Length Polymorphism (Liu et al., 1997)). However, these techniques have still been criticized because the "universal PCR-primers" used for the PCR amplification of the 16S or 23S rRNA genes may not amplify fragments from all community members with equal efficiency (Pepper and Pillai, 1994). Nevertheless, such approaches provide a significant improvement over the 0.1 – 1 % of the community accessible with standard culture-based techniques.

The present research examined another PCR-based DNA fingerprinting approach as a means of comparing microbial communities, and focused on the development of a community-level technique that uses very small quantities of DNA and has no need for culturing or the use of selective PCR primers such as those used in rDNA studies. In particular, RAPD (Randomly Amplified Polymorphic DNA (Williams et al., 1990)) was used to compare a number of aquatic microbial communities and quantify their overall similarity. RAPD employs short primers of arbitrary sequences to amplify random portions of the sample DNA by PCR. Since each primer is short, it will anneal to many sites throughout the target DNA; a fragment is amplified whenever two of these primers anneal close enough together and in the proper orientation with respect to one another. In individuals that have different sequences, the primers will anneal in different places, and therefore produce a different spectrum of fragments from the PCR – a different genetic "fingerprint". Because each primer generates relatively few (5 to 15) distinct bands when separated on an agarose gel, several reactions must be run, using several different

primers, and the results combined to obtain the desired number of markers. Pooled results can then be compared between samples and percent similarity computed. Using multiple primers also helps ensure that a sufficiently large region of the target DNA is scanned when an estimate of overall variance between samples is desired (Ogram and Feng, 1997). Typically, 10 to 15 primers (~ 100 bands) are required for statistical comparison of samples using RAPD markers (Demeke and Adams, 1994; Xia et al., 1995).

RAPD is commonly used to differentiate among closely related strains of bacteria (Busse et al., 1996; Chachaty et al., 1994; Lin et al., 1996; Makino et al., 1994), and use of this technique for mixed genome samples has been suggested, though experimental applications are rare (Bruce et al., 1992; Moll et al., 1998; Picard et al., 1992; Xia et al., 1995). Previous work with individual genome samples has shown that RAPD provides results that are very consistent with other molecular genetic techniques including RFLP (Karp et al., 1996; Paffetti et al., 1996), AFLP – Amplified Fragment Length Polymorphism (Powell et al., 1996), and DNA hybridization (Tanaka et al., 1994).

In this research, the usefulness of RAPD for comparing microbial communities was evaluated in two aquatic systems. The first was a pair of tidal creeks (Hungars Creek and Phillips Creek) that were compared twice during the summer of 1997 (June and July). The second application examined the groundwater microbial communities from anaerobic and aerobic zones of a shallow coastal plain aquifer on Virginia's eastern shore.

2.2. Materials and methods

2.2.1. Site descriptions

2.2.1.1. Tidal creeks

Creek water samples were collected from the Virginia Coast Reserve Long Term Ecological Research site (VCR-LTER) on Virginia's eastern shore. Two marsh creeks, one on each side of the Delmarva Peninsula, were sampled during the summer of 1997 (June and July). Phillips Creek, on the seaside of the peninsula, contains an extensive marsh system (135 ha) while Hungars Creek, on the Chesapeake Bay side, is much wider and shallower with a smaller area of marsh (35 ha). The two creeks are separated by less than 7 km and, consequently, may be compared without major differences in regional or local climate, land-use patterns, or tidal amplitude (MacMillin et al., 1992). A number of biological and physical-chemical parameters differ between these two creek systems including higher organic matter, bacterial biomass, abundance, and productivity in Hungars creek and greater nutrient concentrations (e.g., phosphate, ammonia, nitrate, and nitrite) in Philips Creek (MacMillin et al., 1992). The dissolved organic carbon (DOC) source also differs for the two systems; Hungars Creek's DOC is primarily derived from phytoplankton while marsh grass/detritus is the main DOC source for Phillips Creek.

2.2.1.2. Groundwater

The groundwater samples for this study were collected from a shallow coastal plain aquifer near Oyster, Virginia. The research site occupies an abandoned agricultural field (1.7 ha) that overlies distinct regions of anaerobic and aerobic groundwater.

Samples were collected in August 1997 from four wells, two aerobic (B3 and C3) and two anaerobic (D1 and W2). The groundwater chemistry between these two regions of the field differs substantially, primarily in the amount of dissolved oxygen, alkalinity, nitrate, ammonia, and dissolved iron present (Knapp, 1997). Average microbial abundance also differs between the wells (D1: 5×10^6 cells ml^{-1}; W2: 3×10^7; C3 and B3: 3×10^5 (Lancaster and Mills, 1995)).

2.2.2. Sample collection

To isolate the microbial community for analysis, water samples were filtered onto 0.22 μm pore-size polycarbonate membranes after pre-filtration through AE glass-fiber filters (which removed particulate matter and eukaryotic organisms). Five and twenty liters of water were filtered for the groundwater and creek samples respectively. The DNA from the creeks was collected in conjunction with another research project, so a larger volume of water was sampled at that site. At both sites, the amount of DNA collected was far in excess of that necessary for RAPD community profiling (see below). For the groundwater sites, three replicate five-liter fractions were collected in the field so that within-well and between-well variance could be compared. All filters were transported to the laboratory on dry ice and stored at -70°C until the DNA was extracted.

2.2.3. Extraction of DNA

2.2.3.1. Recovery of cells from filters

DNA from the creek sites was extracted directly from the filters (Fuhrman et al., 1988), quantified using UV spectroscopy, and stored at - 20°C. For extraction from the groundwater samples, bacteria were recovered from the filters by vortex mixing and repeated centrifugation prior to extraction. Specifically, the membranes were cut with scissors into small pieces using aseptic technique, resuspended in ~30 ml of sterile water, and vortex-mixed for 2 min. The supernatant was collected and the filter pieces were resuspended and mixed a second time. The two liquid fractions were pooled and filtered through an AE glass-fiber filter to remove small pieces of the membrane. The bacterial suspension was then centrifuged in two 50-ml centrifuge tubes at 4°C at 30,000 × g for 30 min. All but 5 ml of the supernatant were discarded; the tube contents were resuspended and the liquid from the two tubes combined. This consolidation procedure was repeated several times as necessary until the cell suspension could be transferred into a single 1.5-ml microcentrifuge tube. Acridine orange direct counts (Hobbie et al., 1977) were performed on this suspension in order to normalize the number of cells that entered the DNA extraction procedure, which provided a means of standardizing the amount of DNA entering each PCR reaction.

2.2.3.2. DNA extraction from groundwater cell suspensions

Depending on the concentration of cells in a given suspension, different volumes were removed corresponding to a total of approximately 5×10^7 cells. Cell pellets were collected from these different volumes by centrifuging in a microcentrifuge for 5 min at 14,000 rpm. After discarding the supernatant, 500 µl of buffer (0.02 M Tris (pH 7.8), 0.02 M EDTA (pH 8.0), 0.5 % sodium dodecyl sulfate) was added and the pellet resuspended by vortex mixing. A boiling water bath was then used to lyse the cells (5 min), and 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The mixture was then centrifuged for 5 min as described above. The aqueous layer was transferred to a new tube and, after a second phenol extraction, the DNA was precipitated using an equal volume of cold isopropanol (500 µl). The DNA was centrifuged for 2 min and the pellet was washed twice with ice cold 70% ethanol. The DNA was dried, resuspended in 25 µl of sterile, filtered, deionized water, and stored at – 20 °C.

2.2.4. RAPD amplification

2.2.4.1. Amplification conditions

The PCR reactions were performed using the protocol from Williams et al. (1990) with slight modification. Specifically, 5 µl of the template DNA was added to a 20 µl reaction mixture containing: 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM primer, and 1 unit of *Taq* DNA polymerase. Amplification was performed in a Perkin Elmer 480 DNA Thermal Cycler

programmed for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. Amplification products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed under UV light.

2.2.4.2. DNA template concentration

For the tidal creek samples, several dilutions of the original DNA template were tested to determine the appropriate DNA concentration for the PCR reaction. Serial dilutions of the original stock were prepared in sterile, filtered deionized water, and 10^{-3}, 10^{-4}, and 10^{-5} dilutions all generated similar profiles; this trend was confirmed for five different primers. A 10^{-4} dilution, which corresponds to 225 picograms of DNA per PCR reaction, was used in all subsequent amplifications of the creek community DNA. For each creek DNA sample, three replicate PCR reactions were performed in order to assess the repeatability of the RAPD procedure.

For the groundwater samples, 10^{-2}, 10^{-3}, 10^{-4} serial dilutions of the original DNA stock all produced similar RAPD fingerprints (screened using five primers), and a 10^{-3} dilution was used in all further reactions. This dilution corresponds to amplification of DNA extracted from 2000 cells or an original sample volume from the well of 0.24 μl (using the average bacterial concentration of the four wells = 8.9×10^6 cell ml^{-1}). Additionally, since the 10^{-4} dilution produced similar profiles, it is possible that as few as 200 cells (equivalent to an original sample volume of 0.02 μl) could be used in each RAPD reaction.

2.2.4.3. Primer selection

Forty different primers (purchased from Operon Technologies, Alameda, California) were tested for their ability to provide readily interpretable and reproducible RAPD profiles. Fourteen of these were selected for use in this study (Table 2.1). Though the choice of primers is somewhat subjective, criteria such as number of bands produced, clarity and distinctness of bands, and reproducibility of the RAPD fingerprints were used in the selection process. Another criterion that should be considered when selecting a primer is its ability to detect differences among the samples of interest; a primer that gives identical band patterns for all samples does not help discriminate among communities. This final criterion did not significantly influence primer selection in the present study as 39 of the 40 primers screened showed major differences when tested on groundwater samples from a pair of wells (W2 (anaerobic) and C3 (aerobic)).

From the 40 primers screened, 11 were chosen for use with the groundwater samples. With these, 80 different amplification products (fragments) were observed, of which 91% were polymorphic. Individual well samples contained between 29 and 49 of these bands.

For the creek samples, the same 11 primers were tested and nine of them successfully applied. Three additional primers, not tested on the groundwater samples, were also chosen for use with creek samples. These 12 primers produced a total of 101 distinguishable bands, and an individual sample contained between 44 and 53 of these bands. Of these 101 bands, 92% were polymorphic.

Table 2.1. Sequences and average number of bands generated for RAPD profiling of creek and groundwater samples. "NC" indicates when data were not collected using a given primer.

Operon I.D.	Sequence (5' to 3')	Creeks (# bands)	Groundwater (# bands)
C 4	CCGCATCTAC	6	5
D 5	TGAGCGGACA	7	13
F 4	GGTGATCAGG	13	14
F 7	CCGATATCCC	7	NC
F 5	CCGAATTCCC	6	NC
F 3	CCTGATCACC	9	4
F 1	ACGGATCCTG	5	7
F 14	TGCTGCAGGT	6	NC
S 10	ACCGTTCCAG	10	5
S 13	GTCGTTCCTG	13	6
S 14	AAAGGGGTCC	NC	7
T 7	GGCAGGCTGT	7	5
X 5	CCTTTCCCTC	12	5
X 16	CTCTGTTCGG	NC	9

2.2.5. Analysis of DNA fingerprints

Each fragment visualized on the agarose gels was treated as a unit character, scored as present or absent in each sample, and used to construct a distance matrix and dendrogram based on the Jaccard coefficient, which calculates the proportion of positive bands shared by each sample pair (Sneath and Sokal, 1973). In order to assess the statistical significance of the groupings and subgroupings in each dendrogram, a bootstrapping procedure was employed (Stackebrandt and Rainey, 1995; Swofford et al., 1996). This technique randomly resamples the data, alternately truncating or rearranging the original data set, and computes the fraction of times that a branching point appears in the recomputed trees. This is repeated a number of times (usually between 100 and 1000), and the larger the reported bootstrap value (the proportion of times a particular node appeared in the recomputation process), the greater the significance of the branching point.

In this research, bootstrapping was accomplished by first using "SeqBoot" in the PHYLIP computer program to bootstrap the presence/absence data matrix 100 times (Felsenstein, 1993). Each of the resultant data sets was then fed into the clustering program of SPSS (Version 7.5.1) and similarity matrixes were determined using Jaccard's coefficient. Next, distance matrices (Distance = 1 – Similarity) were computed, and the "Neighbor" program of PHYLIP was used generate a 100 different recomputed trees using UPGMA clustering (Norusis, 1994). The "bootstrap value," the proportion of recomputed trees that contain a give node, was then determined by feeding the tree file from "Neighbor" into the "Consense" subroutine of PHYLIP. In addition to

cluster analysis, principal component analysis (SPSS, Version 7.5.1) of the original data was performed, and diagrams of the first two principal components were constructed. Though principal components analysis is not necessarily appropriate for use with binary data such as these, it is often used as a supplemental means of visualizing the relationships from RAPD profiles (Demeke and Adams, 1994).

2.3. Results and Discussion

The results of the present study suggest that RAPD is a useful technique for studying variation among microbial communities. For the creek water samples, RAPD showed distinct differences between the two sites and the two times (Figures 2.1 and 2.2). For the groundwater samples, differences between the anaerobic and aerobic zones of the aquifer could be distinguished (Figures 2.3 and 2.4). In both investigations, the RAPD results correlated well with differences in the physical-chemical properties of the various sampling sites.

2.3.1. Tidal creeks

Hungars Creek and Phillips Creek were compared twice during the summer of 1997 (June and July), and Figures 2.1 and 2.2 show the results of the principal components and cluster analysis, respectively. In general, temporal changes in community structure were less for Hungars Creek than for Phillips (Hungars Creek – 46% similar for June/July, Phillips Creek – 38%); overall, the two creeks were 30% similar to each other.

The reproducibility of the RAPD procedure was examining by profiling each creek sample three times (three PCR reactions using the same DNA template). PCR repeatability was quite high with replicate community profiles averaging 89% similar (101 bands compared). Additionally, replicate reactions always clustered together in the dendrogram (Figure 2.2) and grouped tightly on the principal component plot (Figure 2.1). One of the major problems reported by users of RAPD is artifactual variation in banding patterns for an individual DNA sample (Ellsworth et al., 1993; Meunier and Grimont, 1993), though the magnitude of this problem varies greatly between laboratories (Penner et al., 1993). The results presented here suggest that, with careful standardization of reagents and amplification conditions, the impact of PCR artifacts on overall community profiles is negligible.

The tree structure presented in Figure 2.2 was analyzed using the bootstrapping procedure described above, and all nodes were very well supported (found in > 65% of the recomputed trees). Bootstrapping not only provided a measure of the support for each cluster, it also confirmed that the RAPD procedure, as applied, generated enough bands to adequately describe the relationship among the samples; random subsampling of the dataset did not significantly alter the observed pattern.

Based upon previous work comparing the bacterial dynamics in these tidal creeks (Lowit et al., 1998; MacMillin et al., 1992), it is not surprising to find that the microbial communities in Hungars and Phillips creeks do differ structurally. Though separated by only a few kilometers, the differences in nutrient concentration, organic matter content,

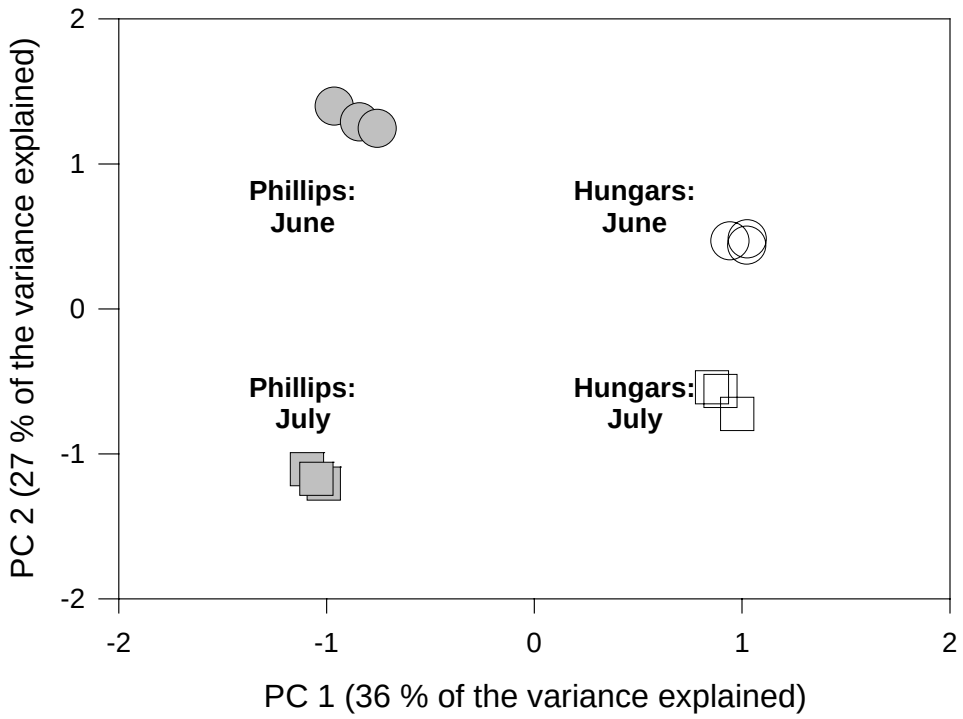

Figure 2.1. PCA of the RAPD profiles for the creek samples. The three points for each site represent replicate PCR reactions of the same DNA template. The percent of variance explained by each PC is listed.

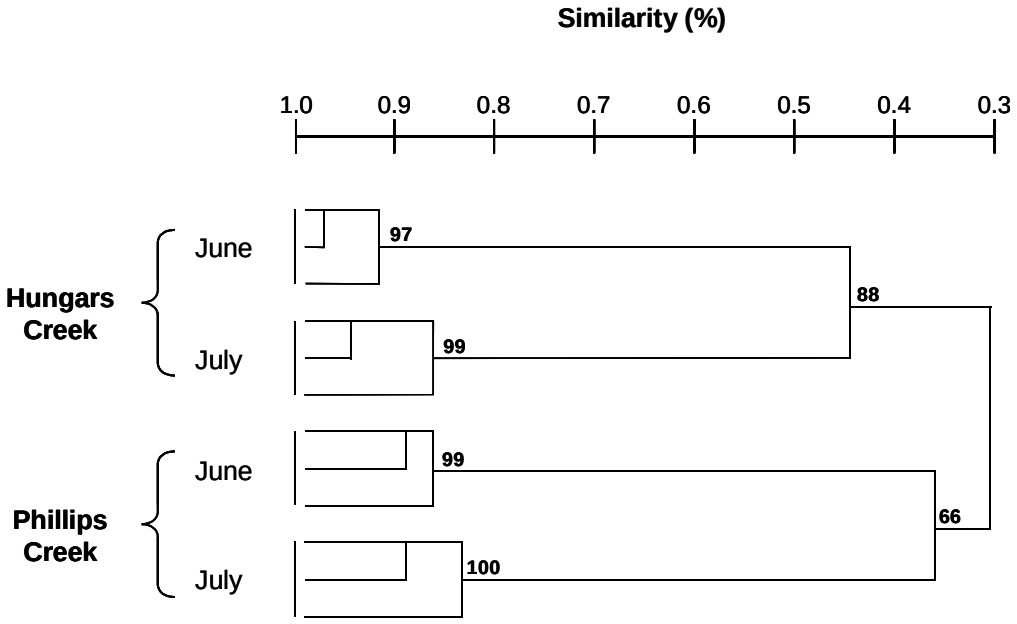

Figure 2.2. Dendrogram displaying the results of a cluster analysis using Jaccard's coefficient as a measure of genetic similarity among the creek samples. The scale along the top represents percent similarity; the three prongs for each site represent replicate PCR reactions of the same DNA template. The numbers at each node are the bootstrap values (bootstrap performed using 100 replications).

and DOC source could support different microbial inhabitants; the two creeks were only 37 % similar in June and 30 % similar in July. MacMillin et al.'s (1992) work also noticed significant differences in bacteria abundance between June and July, and differences in environmental regime between the two months (i.e., elevated nutrient availability and higher water temperatures in July) suggest that community structure may differ temporally. It is especially interesting to note how the different samples were separated by the two major principal components (Figure 2.1); principal component 1 consisted of bands that separated the creek communities by location, and principal component 2 consisted of bands that separated the creek communities by time.

2.3.2. Groundwater samples

Similar statistical analyses were performed to analyze the RAPD profiles for the groundwater sites. Aerobic wells (C3 and B3) were more similar to one another than to either of the anaerobic sites (Figures 2.3 and 2.4). Though the variability among replicate well samples was larger than for the creek samples, the dendrogram (Figure 2.4) still showed a clear separation among wells that was consistent with the observed patterns in groundwater chemistry at this site (Knapp, 1997; Lancaster and Mills, 1995). Historically, well W2 displays a very unique pattern in groundwater chemistry compared to the other anaerobic wells (e.g., higher DOM, lower dissolved iron, and methane emission (Mills et al., unpublished data), and it was the most unique well using RAPD profiling. Similarly, the aerobic wells have nearly identical water chemistry, are physically separated by only 10 m, and clustered tightly as anticipated.

A bootstrapping procedure was again used to determine how well supported the tree topologies were (Figure 2.4); these bootstrap values were generally lower than for the creek water samples, indicating that the overall differences displayed in the dendrogram of the wells were not as significant as those for the tidal creek. Confidence in this tree structure may be improved by increasing the number of RAPD bands compared; for the groundwater samples only 80 bands were used, as opposed to 101 for the creek samples. Greater separation and more stable clusters might have been obtained by using a larger number of bands in the analysis of the groundwater samples. Other studies have also shown that at least 100 bands are necessary when making RAPD comparisons such as this (Demeke and Adams, 1994).

The three replicas compared for each well represent independent samplings (separate 5-liter fractions of water) and show a much greater variability than was observed for the creek samples. It is important to recall, however, that these three values reported for each of the creek communities are replicate PCR reactions from a single DNA sample, and do not describe any of the within-site variability present in these tidal creeks, nor do they reflect any of the variability from the DNA extraction procedure. The within-site variance for the groundwater samples was slightly higher than anticipated and the influence of small-scale spatial heterogeneity within the aquifer may partly explain this. Although an attempt was made to empty the wells of any stagnant water before sample collection, insufficient purging could mean that each fraction of water collected represented a slightly different community experiencing different environmental conditions. At each well, the most unique replicate was usually the first sample collected

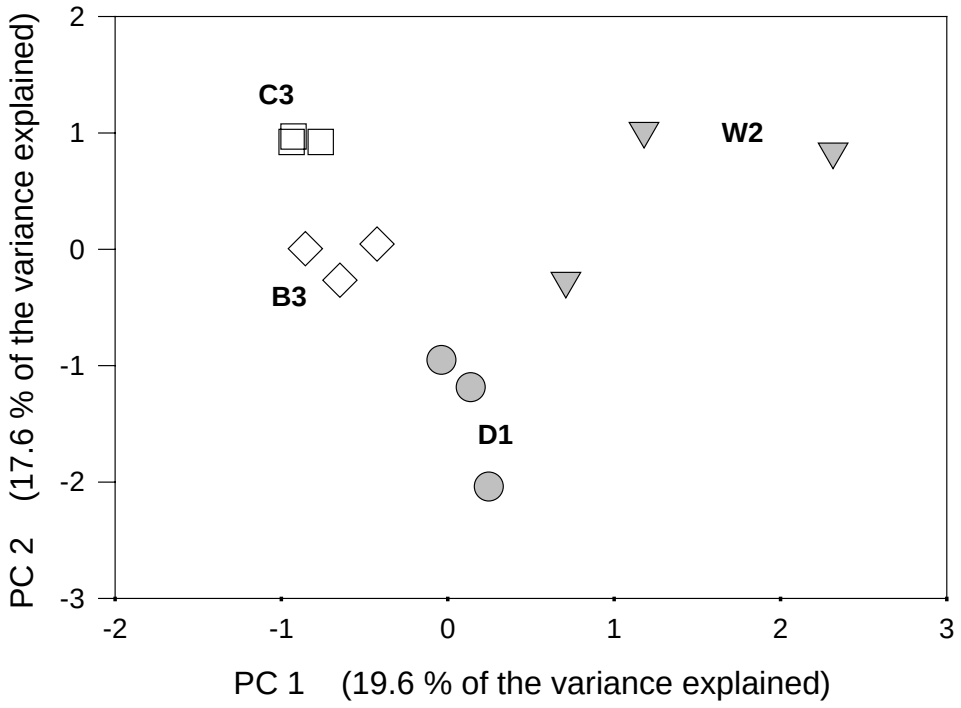

Figure 2.3. PCA of the RAPD profiles for the groundwater samples. The three points for each site represent independent replicas (separate 5-liter fractions of water from each well). The percent of variance explained by each PC is listed.

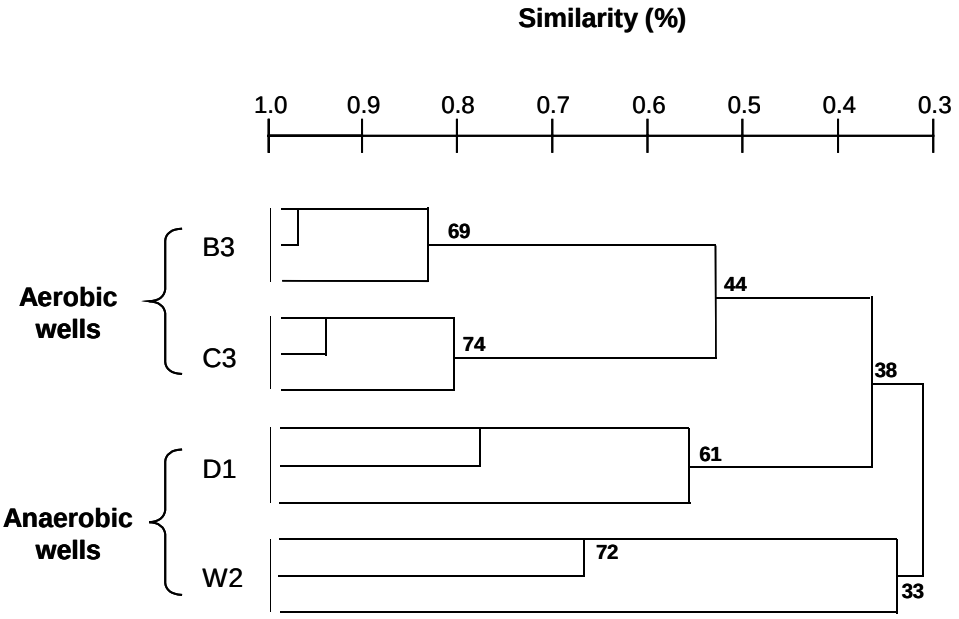

Figure 2.4. Dendrogram displaying the results of a cluster analysis using Jaccard's coefficient as a measure of similarity among the groundwater samples. The scale along the top represents percent similarity; the three prongs for each site represent independent replicas (separate 5-liter fractions of water from each well). The numbers at each node are the bootstrap values (bootstrap performed using 100 replications).

(3/4 times), suggesting that the order of collection, and hence proximity of the sample water to the well opening and air-water interface, was important. It is easy to imagine that different fractions of well water could support different microbial communities in different subhabitats. The fact that the anaerobic wells showed a greater within-site variance relative to the aerobic wells (Figures 2.3 and 2.4) helps confirm this, as it is anticipated that within-well differences in oxygen availability would be more influential under hypoxic conditions.

One of the most promising aspects of the RAPD technique was the small quantity of DNA required and the ease of the DNA extraction procedure. The extraction procedure used with the groundwater samples was a simpler, more rapid, alternative to the Fuhrman et al. (1988) approach used in the creek water samples, and its successful use in this study demonstrates that relatively crude DNA preparations may be used to profile communities with RAPD. Though more variability was observed for replicate PCR reactions of the same groundwater sample (results not shown) compared to the repeated PCRs of a single creek sample (Figure 2.2), the contribution of this variability to the overall variance within the groundwater wells was observed to be quite small.

Considering the small amount of DNA used in the PCR reactions, it is possible that smaller volumes of water could be collected for an analysis of a microbial community. Our calculations suggest that enough DNA could be obtained from as few as 3×10^4 cells (which corresponds to ~ 3.5 μ l [0.24 μl × 15 primers] from each of the groundwater wells in this study). In theory, even smaller samples are possible; PCR has been used to amplify DNA from a single cell (Davis et al., 1994). Needless to say, such

extremely small samples will not capture the genomic diversity that exists in natural communities. The implementation of RAPD as a means of studying microbial community structure means that sample size, and the spatial scale over which variability can be studied, are no longer limited by technical considerations. Using more traditional techniques, the measurements made of microbial community structure typically represent broad-scale characterizations, and rarely consider the small scale on which the populations may actually be interacting. However, the uneven distribution of microorganisms in the environment suggests that community structure may vary intensely at small spatial scales, depending upon heterogeneities in the environmental matrix and localized nutrient availability. The very small sample size required for RAPD profiling of communities offers a means of examining the spatial scale of the variance in community structure that has not previously been available. Ultimately, a better understanding of this natural variation will help microbial ecologists as they try to make inferences about how structure influences other community-level characteristics.

2.4. Conclusions

The results of the present study confirm that RAPD fingerprinting is a very useful means of comparing microbial communities. Analysis of two separate aquatic systems provided results consistent with historical knowledge of the sites' different environmental regimes. The RAPD approach has many advantages over other DNA-based, community-level analyses including the fact that it does not involve culturing, is very rapid, and is simple to perform. The primers used in RAPD are not selective for specific organisms,

groups of organisms, or particular genes, and can therefore provide a better representation of the entire community than more traditional PCR-based approaches (e.g., 16S rRNA). Many RAPD primers kits are commercially available (e.g., Operon Technologies, Genosys Biotechnologies) making primer screening and selection very easy. As with other PCR-based techniques, RAPD uses very small quantities of DNA, decreasing sample collection time and permitting the analysis of fine-scale spatial patterns in microbial community structure. Larger-scale changes may also be monitored if appropriate sample volumes are used. PCR's suitability to small, mixed, degraded, and impure samples make it especially useful in environmental applications (Bej and Mahbubani, 1994), and this research suggests that relatively crude DNA extracts may be used, as was done with the groundwater samples, further increasing the speed with which a community analysis can be performed. Moreover, the ease of RAPD analysis means that an appropriate number of replicates may be considered using this procedure; with most other techniques the practical limits of replication fall short of what is necessary for adequate statistical significance.

The primary disadvantage to RAPD analysis is the large number of primers and PCR reactions that much be performed in order to obtain enough bands for statistical comparison of the community profiles. In response to this, we are investigating the application of other PCR-based DNA fingerprinting techniques (e.g., AFLP (Zabeau and Vos, 1993), in the analysis of microbial communities. Moreover, it is not clear at this time how sensitive the RAPD technique is at detecting rare organisms within bacterial communities, and this must be considered as one draws conclusions from RAPD data.

Some have estimated the lower limit of detection to be 1% of the total DNA pool (Xia et al., 1995), but it is likely that this ratio will vary somewhat depending upon the overall complexity of the community.

While RAPD fingerprinting is a useful technique for comparing communities without the limitations imposed by culture dependent procedures, it does not provide any direct information about the constituent members of the community being examined. But, other community-level analyses (e.g., DNA hybridization) cannot provide such information either. Other approaches (e.g., 16S rDNA) can provide some information about the presence or absence of some strains, but no technique exists to completely characterize the structure of bacterial communities. However, whole-community studies such as these are the only means of holistically examining microbial systems and evaluating how the entire suite of organisms responds to changing environmental parameters. In combination with more traditional approaches, DNA fingerprinting techniques may allow scientists to move beyond their inability to completely categorize the constituents of a community toward the development of a more complete understanding of the overall interactions among bacterial populations and between populations and the environment.

Chapter 3.

The impact of dilution on microbial community structure and functional potential: a comparison of numerical simulations and batch culture experiments.

Franklin, R. B., C. H. Bolster, J. L. Garland, and A. L. Mills. 2001.
Applied and Environmental Microbiology. 67:702-712.

Abstract

A series of microcosm experiments was performed using serial dilutions of a sewage microbial community to inoculate a set of batch-culture experiments in sterile sewage. After inoculation, the dilution-defined communities were allowed to regrow for several days, and a number of community attributes were measured for the regrown assemblages. Based upon a set of numerical simulations, community structure was expected to differ along the dilution gradient; the greatest differences in structure were anticipated between the undiluted/low dilution communities and those regrown from the very dilute (more than 10^{-4}) inocula. Furthermore, some differences were expected among the lower dilution treatments (e.g., between undiluted to 10^{-1}) depending upon the evenness of the original community. In general, each of the procedures used to examine the experimental community's structure separated the communities into at least two, often three, distinct groups. The groupings were consistent with the simulated dilution of a mixture of organisms with a very uneven distribution. Significant differences in community structure were detected with genetic (AFLP and T-RFLP), physiological (CLPP), and culture based (colony morphology on R2A agar) measures. Along with differences in community structure, differences in community size (AODC), composition (ratio of sewage medium counts to R2A counts, monitoring of each colony morphology across the treatments), and metabolic redundancy (i.e., generalist versus specialist) were also observed, suggesting the differences in structure/diversity of communities maintained in the same environment can manifest as differences in community organization and function.

3.1. Introduction

Ecological diversity, the variety and abundance of species in different habitats and communities, is one of the central themes of ecology. Diversity is commonly thought to be a useful indicator of the well-being of an ecological system; however, there is considerable debate in the literature over the role diversity plays in ecosystem function (Chapin et al., 1997; Lawton, 1994; Magurran, 1988; McNaughton, 1977; Naeem et al., 1994; Pimm, 1984; Putman, 1994; Rosenzweig, 1995). Most of this uncertainty arises from the practical limitations of measuring and manipulating diversity for experimental studies. Testing the effects of diversity on any community property or ecosystem function requires knowledge of the diversity of the community under examination; however, there are no methods currently available that allow microbial diversity to be measured. Numerous procedures are available for monitoring changes in community structure (e.g., culture-based analyses, community-level physiological profiling (CLPP), analysis of the lipid contents of microbial cells, and molecular genetic techniques), and these approaches each have biases and limitations that are well documented (for review articles, see: Bej and Mahbubani (1994), Busse et al. (1996), Garland (1996), Griffiths et al. (1997), Holben (1997), Ogram and Feng (1997), Stahl (1997), Torsvik et al. (1994), and White et al. (1997)).

Despite the inability to measure diversity directly, Garland (Garland and Lehman, 1999; Garland et al., 1999) and Morales et al. (1996) successfully used “dilution” to manipulate microbial diversity for several applications. The premise behind these studies was that dilution of a relatively diverse community would remove rare organism types, creating mixtures of cells differing in species richness. Regrowth of these diluted

mixtures should then produce cultures of roughly the same biomass, but differing in overall diversity. In these studies, the various dilution/diversity communities responded differently to invasion attempts (Garland et al., 1999; Morales et al., 1996) and to environmental stress (Garland et al., 1999), with more diverse (less dilute) communities being more stable and better able to withstand invasion. However, with no good way to evaluate or quantify microbial diversity, the magnitude of the diversity differences being evaluated remains unknown.

The present work sought to define the relationship between dilution and resultant changes in diversity and community structure. First, several numerical simulations were performed in order to develop a set of expectations about how overall diversity (expressed as the Shannon-Wiener index), richness (number of species or types of organisms in the community), and evenness (the relative distribution of individuals among these types) change with dilution. Next, a series of microcosm experiments was conducted using batch cultures of sterile sewage, inoculated with serial dilutions of fresh sewage. After regrowth of these batch cultures, several methods were used to characterize the microbial communities, including traditional microbiological procedures, CLPP, and molecular genetic techniques. The regrown communities differed along the dilution gradient, and the results followed a pattern similar to that observed in the simulated dilution of a relatively uneven mixture of organisms. The results of this work will be useful in planning future studies as the ability to create natural communities systematically differing in complexity could allow researchers to manipulate diversity, perhaps in a quantifiable way, while evaluating its relationship to other community-level properties (e.g., stability, invasibility, or spatial heterogeneity).

3.2. Materials and methods

3.2.1. Numerical simulations

To examine the theoretical effect of dilution on community inoculum composition, a series of numerical simulations (coded in MATLAB©) were performed. Communities were constructed by assigning each of 10^6 individuals a random species identification based on a normal distribution of integers from 1 to 1000. The mean of this distribution was set at 500; the variance was adjusted in order to create communities of different initial evenness (Figure 3.1 A). Variance levels of 100, 250, 1000, and 20,000 were used, and a perfectly even initial community was also generated (1000 types, each containing 1000 individuals). Dilution of each of these five initial communities was simulated by randomly selecting 1/10th of the individuals from the array representing the undiluted community. The species identification of each individual in this subset was copied to a second array, which served as the initial community for the next dilution in the series (dilutions extended through 10^{-5}). For each community, at each dilution level, richness, evenness, and diversity were calculated. Richness (S) was taken to be the number of organism types (species) in the community. Diversity was expressed as the Shannon-Wiener index (H'):

$$H' = \sum_{1}^{i} p_i \ln p_i$$

where i indicates each species or category and p_i is the proportion of individuals of each species (Shannon and Weaver, 1949). Evenness (E) was calculated as $E = H'/H'_{max}$, where $H'_{max} = \ln S$ (Pielou, 1969).

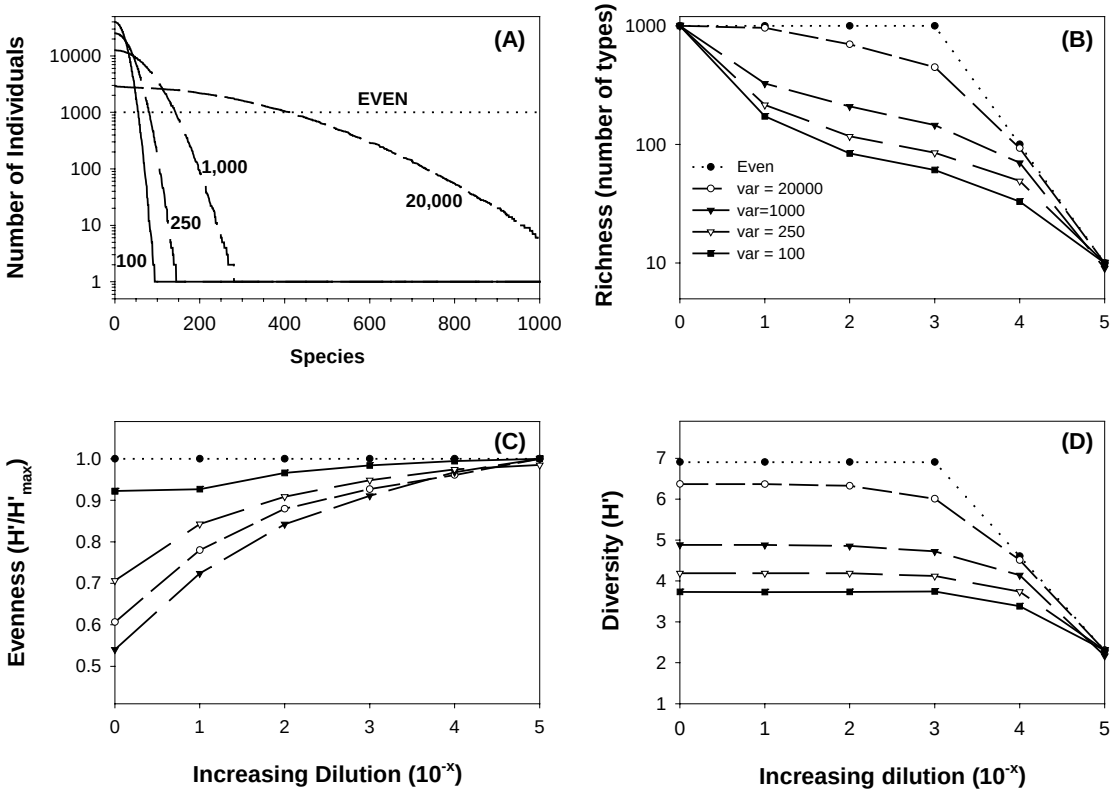

Figure 3.1. Results of the numerical simulations of the effect of dilution on community diversity, richness, and evenness. (A) The distribution of individuals among 1000 types for the initial communities used in the simulations; note that both total abundance and richness were the same in each of the five communities. The mean of each distribution was set at 500, and the variance was altered to simulate communities with a dominant (var = 100, 250, or 1000) or relatively even (var = 20,000 or even) distribution. (B), (C), and (D) are the simulation results and show how community structure differed in the various initial communities for each serial dilution. The X-axis represents the negative exponent of the dilution factor (e.g.,"4" corresponds to a 10^{-4} dilution) and the Y-axis represents: (B) richness (number of types or species), (C) evenness, and (D) the calculated value of the Shannon-Wiener diversity index.

3.2.2. Batch culture experiments

3.2.2.1 Microcosm setup

Raw sewage was collected from the Cape Canaveral Air Station Wastewater Treatment Facility (Kennedy Space Center, FL) and used as the inoculum for the microcosm experiments. A single large sample (2 L) was collected from the equilibration basin and allowed to settle for approximately two hours to remove large particles. A 10-fold serial dilution (though 10^{-6}) of the supernatant was prepared in autoclaved sewage; each of these different dilutions then served as an inoculum for the batch culture incubations. Before dilution, the concentration of cells in the supernatant, determined by acridine orange direct counting (AODC (Hobbie et al., 1977)), was 1.8×10^{6} cells ml^{-1}.

The batch cultures were established by adding 1 ml of inoculum to 60 ml of autoclaved sewage in a 125-ml Erlenmeyer flask. Seven treatments (10^{0} through 10^{-6}) were maintained in this experiment, with three replica communities at each dilution. All flasks were capped with sterile foam plugs to prevent contamination and kept on a shaker table, operated at 150 rpm, to maintain aerobic conditions. Each day, 20 ml of liquid was removed from each flask and replaced with 20 ml of sterile sewage. After 9 days (three retention times), the communities were harvested and analyzed.

3.2.2.2. Cultural counts and diversity of colony morphology

For each flask, a serial dilution of the regrown community was plated, in duplicate, onto both R2A agar (Difco, Detroit, MI) and onto sterile sewage media (SM). SM was prepared by mixing 15 g of agar per liter of sterile sewage supernatant. Plates

were incubated at room temperature (ca. 23°C), and the number of colony forming units (CFU) on R2A agar was determined after 3 days. Growth on SM was evaluated after 6 days.

The diversity of colony morphologies on R2A was compared across the different dilution treatments. For each flask, two plates were selected; on each plate, 25 colonies were randomly chosen and colony morphology described based on size, pigmentation, form, elevation, and surface. Richness (number of distinct colony morphologies), evenness, and diversity (Shannon-Wiener diversity index) were then calculated.

3.2.2.3. Community-level physiological profiling

For each flask, a 10^{-1} dilution of the microbial community was prepared (in sterile water) and inoculated into Biolog GN microplates. Plates were then incubated at room temperature (ca. 23°C). Color formation in each of the 96 wells of each plate was monitored by periodically (every 2 to 4 hours) measuring the absorbance at 590 nm using a Biotek EL 320 microplate reader. Data were normalized using a blank-corrected average well color development of 0.75 absorbance units and analyzed using a principal components analysis (PCA) (Garland, 1996; Garland and Mills, 1991).

3.2.2.4. Dilution/extinction analysis of CLPP

Dilution/extinction analysis was performed on a subset of the regrown communities (one replicate flask from each of the undiluted, 10^{-2}, 10^{-4}, and 10^{-6} dilution treatments) to determine the relationship between cell density (I) and functional richness (number of positive wells (R)) (Garland and Lehman, 1999). Serial dilutions of the

microbial suspensions were inoculated into Biolog GN microplates, incubated at room temperature for 7 days, and absorbance at 590 nm was measured. A positive response was defined as any value greater than 0.25 absorbance units (after correction for the control well) and a hyperbolic model, $R = (R_{max} \times I) / (K_I + I)$, where R_{max} equals the maximum (asymptotic level) of R and K_I is the value of I when R is 1/2 of R_{max}, was fit to the data (Garland and Lehman, 1999).

3.2.2.5. Molecular analysis of whole community DNA

3.2.2.5.1. DNA extraction and quantification

At harvest, approximately 40 ml of sample was collected from each flask and the suspended microbial community concentrated by centrifugation (23,000 × g, 20 min). The resultant cell pellet was suspended in 200 µl of phosphate-buffered saline (PBS) and stored at -20°C. Whole-community DNA was extracted using the High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN) and quantified using the PicoGreen dsDNA quantification reagent (Molecular Probes, Eugene, OR).

3.2.2.5.2. AFLP

Amplified Fragment Length Polymorphism (AFLP) was completed using the Perkin Elmer Microbial Fingerprinting Kit (PE Applied Biosystems, Foster City, CA) following the manufacturer's instructions for analysis of individual bacterial strains. Three different pairs of primers, each with a different fluorescent label, were used for the selective AFLP amplification: EcoRI-AA (JOE labeled) with MseI-CA, EcoRI-AC

(FAM labeled) with MseI-CC, and EcoRI-AT (NED labeled) with MseI-CT. For complete primer and adaptor sequences, and for explanation of primer selection criteria, see the *PE Applied Biosystems AFLP Microbial Fingerprinting Protocol.*

Selective amplification products were resolved using an ABI Prism 310 Genetic Analyzer following the manufacturer's instructions with slight modification. For each sample with each primer pair, 1 μl of PCR product was analyzed and the sample injection time was decreased from 12 sec to 10 sec. Data were analyzed using the Genotyper software (PE Applied Biosystems), and the presence or absence of each peak in each sample was coded as 1 or 0. This type of data matrix was prepared for each primer pair, and the information from the three primer pairs pooled into a single large data set. The Jaccard coefficient was used to determine distances between samples (relative similarity), and a cluster analysis (using UPGMA between-groups linkage) was performed. A bootstrapping analysis was then used to assess the significance of each group and subgroup in the cluster analysis (Franklin et al., 1999a).

A PCA was also performed on the original pooled data matrix (SPSS 9.0) and plots of the first two principal components were made. As PCA is not strictly mathematically appropriate for use with binary data, its application in this study was solely to aid in visualization of the relationships among the samples and not in statistical evaluation. Such an approach has been used several times to compare samples profiled using a variety of similar genetic techniques (Franklin et al., 1999a; Franklin et al., 1999b; Wikström et al., 1999; Wikström et al., 2000); PCA generally provides the same information (groupings and relative distances among samples) as the above outlined cluster analysis.

3.2.2.5.3. T-RFLP

Each T-RFLP (Terminal Restriction Fragment Length Polymorphism) reaction mixture (50 μl) contained: 25 ng community DNA, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, each primer at a concentration of 0.1 μM, and 1.25 units of *Taq* DNA polymerase (Liu et al., 1997). The bacterial 16S rRNA gene was amplified using two primers: 1392 Reverse (5'ACGGGCGGTG TGTRC) and 27 Forward (5'AGAGTTTGATC CTGGCTCAG (labeled with 6-FAM)). The PCR program was: 94°C for 3 min, followed by 30 cycles of 94°C (30 sec), 56°C (45 sec), and 72°C (2 min), with an additional final extension at 72°C for 3 min. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI) and eluted in a final volume of 50 μl. Separate portions (10 μl) of the purified PCR product were then digested with either the *Hha*I or *Msp*I restriction enzyme, using the manufacturer's recommended reaction buffer and 20 units of enzyme (New England Biolabs, Beverly, MA). Digests were incubated at 37°C for 4 h.

The lengths of the fluorescently labeled terminal restriction fragments were determined for each sample using the ABI 310 Genetic Analyzer. Three microliter portions of each digested product were mixed with 24 μl of deionized formamide and 1 μl of GeneScan-1000 [ROX-labeled] size standard (PE Applied Biosystems, Foster City, CA), denatured at 95°C for 5 min, and quickly chilled on ice. Electrophoresis was

performed using the same conditions as for AFLP, but with a 40-min run time. Data were analyzed using the GeneScan software (PE Applied Biosystems) with a peak height detection of 100. As with the AFLP analysis, the presence or absence of each T-RFLP in each sample was determined, and the data from each restriction enzyme pooled for cluster, bootstrapping, and principal components analyses.

3.3. Results

3.3.1. Numerical simulations

The results of the numerical simulations show that, while microbiologists generally consider "dilution" to be a linear process, the response of various community-level parameters (richness, evenness, and diversity), to such a manipulation may produce non-linear results (Figure 3.1 B, C, and D). There was no change in diversity (H′) of the even community with dilution until the number of individuals (10^3 individuals) equaled the number of species (10^3 types) at the 10^{-3} dilution. With further dilution, H' decreased in response to the rapid loss in species richness (Figure 3.1 B), though evenness remained 1.0 (Figure 3.1 C). In each of the other initial communities, a similar trend was observed; in general, H' remained constant until the 10^{-3} (var = 250, 1000, and 20,000) or 10^{-4} (var = 100) dilution. After this point, diversity decreased, corresponding to the loss in species richness and an increase in community evenness. At the end of the dilution series (10^{-5}), H' of all the communities decreased to 2.30, which is the maximum theoretical value of H' for a perfectly even distribution among 10 organisms. The sole exception to this was

in the community created by setting var = 1000; here the value of H' at the 10^{-5} dilution was 2.16, because only 9 species were recovered in that particular simulation whereas 10 were recovered in all the others.

The richness of the simulated communities decreased with increasing dilution, but the magnitude of this change varied depending on the initial evenness of the mixture (Figure 3.1 B). For communities of low initial evenness (e.g., var = 100 or var = 250), richness decreased rapidly with the first dilution. For initial communities that were more even, the number of species lost in the first dilution was small; in the perfectly even community, no species were lost. Moreover, the relative distribution of organisms in the perfectly even community did not change upon dilution (Figure 3.1 C). For all other communities, evenness increased with dilution, approaching a theoretical maximum of 1.0 at the 10^{-5} dilution. Maximum evenness was attained in all communities, except for var = 1,000, which, as mentioned above, did not contain 10 species at its final dilution, but only 9.

3.3.2. Batch culture experiments

3.3.2.1. Microscopic and cultural counts

After nine days of regrowth, each experimental community was sampled, and AODC and cultural counts were performed (Table 3.1). An ANOVA was used to determine whether each parameter varied significantly across the different dilution/diversity treatments and a modified LSD (Bonferroni) test was used for multiple comparisons. Total cell concentrations were similar across the first several dilution treatments (10^{0} through 10^{-4}), but in the communities regrown from each of the higher

dilution inoculum (10^{-5} and 10^{-6}), abundance was significantly greater (d.f. = 20, F = 25.7, $p < 0.00001$). Cultural counts on both SM and R2A agar showed a trend similar to the AODC, with significantly greater concentrations of organisms in the higher dilution/lower diversity treatments (R2A: d.f. = 20, F = 4.3, p = 0.0116, SM: d.f. = 19, F = 3.6, p = 0.0259).

Percent culturability on R2A agar (R2A counts divided by AODC) showed a major increase from the undiluted inocula to the 10^{-6} dilution (Table 3.1). The various treatments could be separated into three statistically significant subgroups – group 1: undiluted (10^{0}) through 10^{-4}; group 2: 10^{-3} through 10^{-5}; and group 3: 10^{-6} (d.f. = 20, F = 61.01, $p < 0.00001$). The average percent culturability for each subgroup was: group 1 - 9%, group 2 – 20%, and group 3 – 100%. Percent culturability on SM was also calculated and, though the results were not statistically significant (d.f. = 19, F = 1.97, p = 0.1445), the same general trend was observed. Using the subgroupings defined in the R2A analysis, average percent culturabilty on SM varied as follows: group 1 – 12%, group 2 – 17%, and group 3 – 44%. The average ratio of the SM counts to the R2A counts was also calculated from these data (Table 3.1). For the lower dilution treatments, growth was greater on the sewage medium than on the R2A agar, though the difference between the two was generally not large. However, this trend was reversed in the high dilution/low diversity treatments (10^{-5} and 10^{-6}) where growth on R2A plates was substantially greater than on SM.

Table 3.1. Average of direct and plate count analyses ± one standard deviation (when listed). "Dilution" refers to the original dilution used as the inoculum for each regrown community. Each superscript letter denotes a significantly different group of communities as determined in the text; significance was not evaluated for SM:R2A.

Dilution	AODC ($\times 10^6$ cells ml^{-1})	R2A counts ($\times 10^6$ CFU ml^{-1})	R2A: % culturability	SM counts ($\times 10^6$ CFU ml^{-1})	SM: % culturability	Ratio of SM counts to R2A counts
10^0	22.0 ± 10.0 [a]	0.7 ± 0.4 [a]	4.5 [a]	0.6 ± 0.2 [a]	3.2 [a]	1.1
10^{-1}	13.0 ± 8.6 [a]	0.7 ± 0.5 [a]	5.1 [a]	1.2 ± 0.7 [a]	10.0 [a]	2.1
10^{-2}	24.0 ± 14.0 [a]	1.4 ± 1.2 [a]	7.5 [a]	2.2 ± 1.2 [a]	13.3 [a]	2.2
10^{-3}	30.0 ± 15.0 [a]	4.6 ± 2.7 [a]	14.3 [ab]	5.2 ± 1.7 [ab]	18.3 [a]	1.3
10^{-4}	39.0 ± 2.8 [ab]	5.6 ± 1.7 [a]	14.4 [ab]	5.9 ± 1.6 [ab]	15.3 [a]	1.2
10^{-5}	73.0 ± 19.0 [b]	23.6 ± 11.0 [ab]	32.4 [b]	12.4 ± 3.8 [ab]	18.0 [a]	0.6
10^{-6}	110.0 ± 9.0 [c]	204.0 ± 1.9 [b*]	100.0 [c †]	58.1 ± 83.2 [b]	44.1 [a]	0.8

* On the R2A plates spread from two of the three 10^{-6} treatment flasks, colony growth was too extensive and data were recorded as too numerous to count. The value presented here, and used in all further calculations, was established by assuming a count of 300 CFU on the most dilute plate, for each of those replica flasks, and averaging this with the counts obtained for the remaining flask.

† In the communities regrown from the 10^{-6} dilution, growth on R2A exceeded AODC and so culturability of 100% was inferred.

3.3.2.2. Diversity of colony morphologies

The diversity, richness, and evenness of R2A colony morphotypes varied across the different dilution treatments (Figure 3.2). Because of the high growth that occurred on all plates spread from the regrown 10^{-6} dilution community, it was not possible to evaluate these characteristics for that treatment. However, only three colony types could be distinguished on these plates, and all three were distinctly different from the colony morphologies described in the other treatments. Overall, colony diversity was highest in the communities regrown from the undiluted inoculum, and decreased with increasing inoculum dilution (Figure 3.2 A). The greatest change in colony diversity was observed between the undiluted (10^{0}) and the 10^{-1} regrown communities. Richness decreased along the dilution/diversity gradient, and the most types were also lost after the first dilution (Figure 3.2 B). In general, evenness increased along the dilution/diversity gradient (Figure 3.2 C), though a decrease in evenness was observed between the undiluted (10^{0}) and 10^{-1} dilution treatments.

The distribution of each colony type across each treatment was also examined and 40 % of the colony morphologies encountered in the low dilution/high diversity treatments (10^{0} and 10^{-1} treatments were pooled for this calculation) were not recovered from any of the other regrown communities (Table 3.3). Furthermore, the colony morphologies identified in the highest dilution/lowest diversity (10^{-6}) treatment were all unique. All of the organisms encountered at intermediate dilutions (10^{-2} through 10^{-5}) were also described in either the 10^{0} or the 10^{-1} treatments.

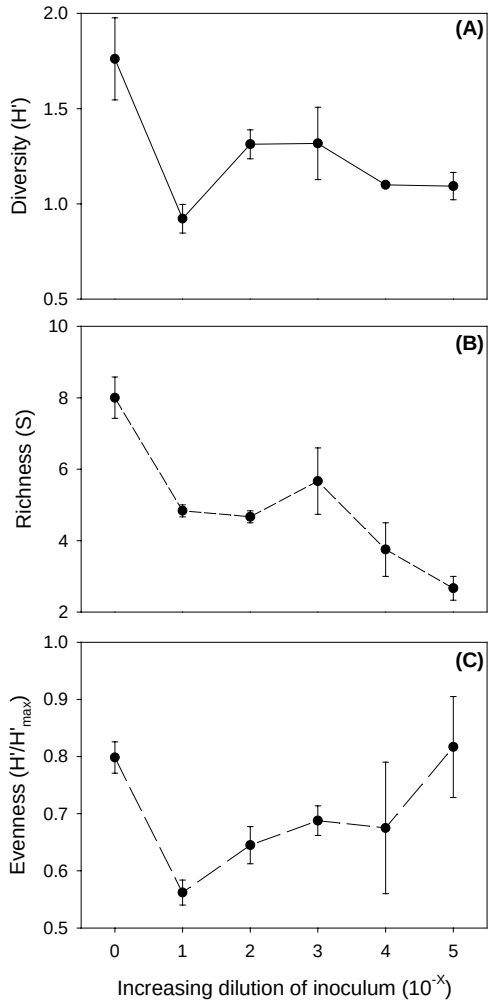

Figure 3.2. Results of the comparison of R2A colony morphology across the different dilution/diversity communities in the batch culture experiments. All values are reported as the average per R2A plate ± one standard error. Each value was calculated by comparing 25 randomly selected colonies on each plate, using two replicate plates per flask and three flasks for each treatment. The sole exception to this was for the 10^{-4} treatment where only two of the replicate flasks were compared. The X-axis in each of these graphs represents the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution). The Y-axis represents: (A) diversity of colony morphologies based upon the Shannon-Wiener diversity index, (B) richness, the number of distinct colony morphotypes, and (C) evenness for each treatment.

3.3.2.3. Community-level physiological profiling

PCA of the CLPP data showed that the various dilution/diversity communities differed in their overall pattern of carbon utilization (Figure 3.3). An ANOVA was performed on the scores from the first two PC's and two homogeneous subsets were established. The communities regrown from the undiluted (10^0) through the 10^{-4} dilution inocula were significantly different from the 10^{-5} and 10^{-6} dilution treatments. This difference was primarily due to variation in the PC 1 scores (d.f. = 20, F = 18.2, $p < 0.00001$); PC 2 did not contribute significantly to this separation (d.f. = 20, F = 2.23, $p = 0.102$).

3.3.2.4. Dilution/extinction analysis of CLPP

The undiluted (10^0), 10^{-2}, 10^{-4}, and 10^{-6} treatments were examined using dilution/extinction analysis of functional characters in the CLPP assays. Plots were made of the number of positive tests obtained in the dilutions made from each regrown community versus the number of cells (AODC) inoculated into each well of the BIOLOG plate (Figure 3.4), and the data were fit with a rectangular hyperbola to estimate the parameters R_{max} and K_I (Table 3.2). R_{max} decreased along the dilution/diversity gradient, however, considering the confidence intervals about these estimates, it cannot be concluded that this decrease was significant. K_I decreased significantly along the dilution/diversity gradient; higher values of K_I were found for communities that were predicted to have a higher diversity based upon extent of dilution.

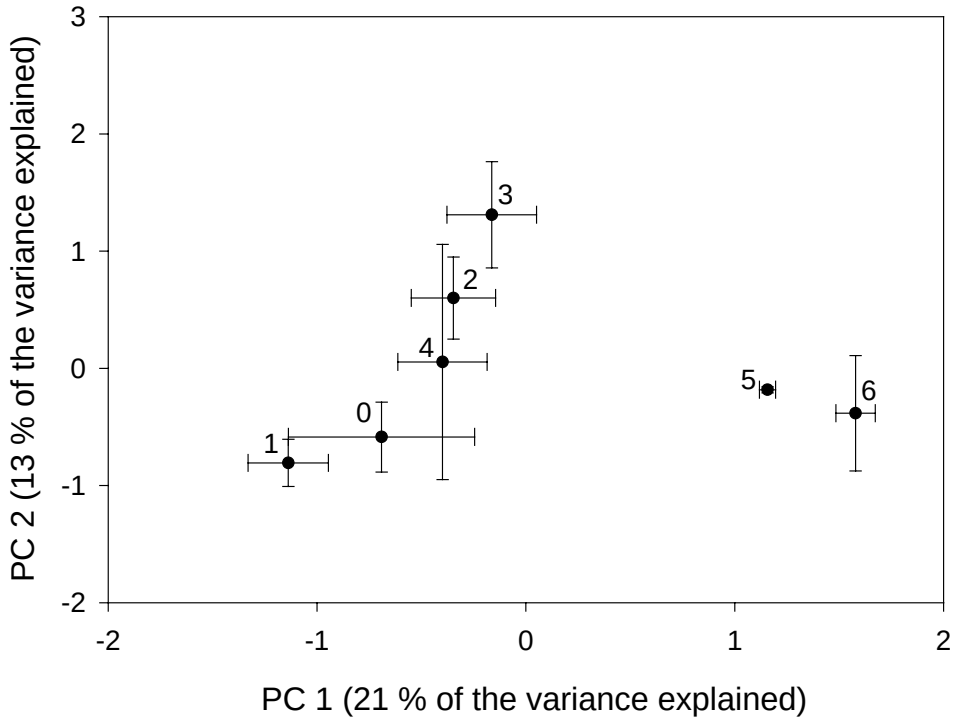

Figure 3.3. Results of a PCA of the CLPP data. Each point represents the average for the three replicate flasks maintained at each dilution; error bars represent ± one standard error. Each treatment is identified by the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution). The percent of variance explained by each principal component is provided.

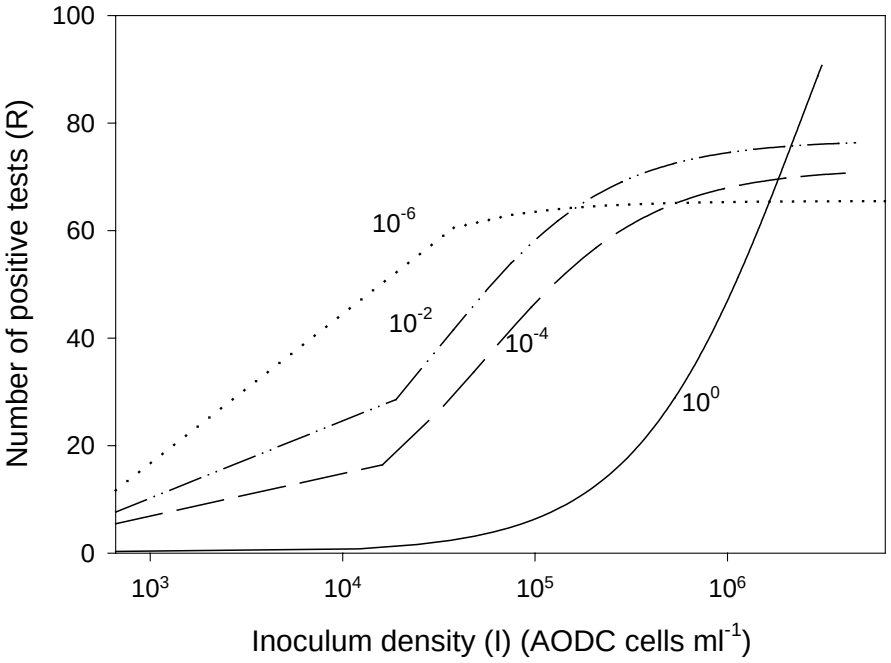

Figure 3.4. Results of the dilution/extinction analysis of CLPP for each regrown community. The X-axis represents the inoculum density (as measured by AODC) used in each CLPP assay (presented on a $\log_{10}$ scale). The Y-axis is the number of positive tests for each incubation. The results are presented as fitted lines, generated by modeling the untransformed data with a right rectangular hyperbola; the associated regression statistics for this fit are given in Table 3.2. The curvature of the regression lines at lower inoculum levels is an artifact of the log scaling of the X-axis.

Table 3.2. Results from the dilution/extinction analysis of CLPP. Errors terms are ± one standard error. "Dilution" refers to the original dilution used as the inoculum for the regrown communities.

Dilution	R_{max}	K_I ($\times 10^4$ cells ml^{-1})	R[a]
10^0	163.5 ± 89.9	250	0.95
10^{-2}	71.6 ± 9.4	5.4 ± 3.4	0.90
10^{-4}	76.9 ± 8.6	3.2 ± 1.5	0.92
10^{-6}	65.5 ± 7.1	0.31 ± 0.2	0.82

[a] Multiple correlation coefficient.

3.3.2.5. AFLP

Combined, the three AFLP primers generated a total of 106 unique PCR fragments. On average, each sample contained 22 fragments; nearly all (90%) of the bands encountered in the low dilution treatments (10^{0} and 10^{-1}) were unique, while 50% of the fragments observed in the high dilution treatments (10^{-5} and 10^{-6}) were not encountered in any of the other treatments (Table 3.3).

Principal component and cluster analyses of the AFLP data showed that the microbial communities in this experiment could be separated into three distinct groups based on overall genetic composition. The communities regrown from the undiluted inocula (and one of the replicas from the 10^{-1} communities) were most unique, a second group was formed from the communities regrown from the "middle dilution" inocula (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and one of the 10^{-5} replicas), and the third cluster included the communities regrown from the very dilute inocula (10^{-5} and 10^{-6}). This pattern is most easily visualized on the PC plot (Figure 3.5), although cluster analysis produced the same separations (results not shown). A bootstrapping procedure (using 100 replications) was performed to assess the significance of the groupings obtained in the cluster analysis (Franklin et al., 1999a). The three clusters outlined above were recovered 93% of the time; this high value suggests that the separation was very well supported by the data and represents a significant difference in overall structure between the three sets of communities.

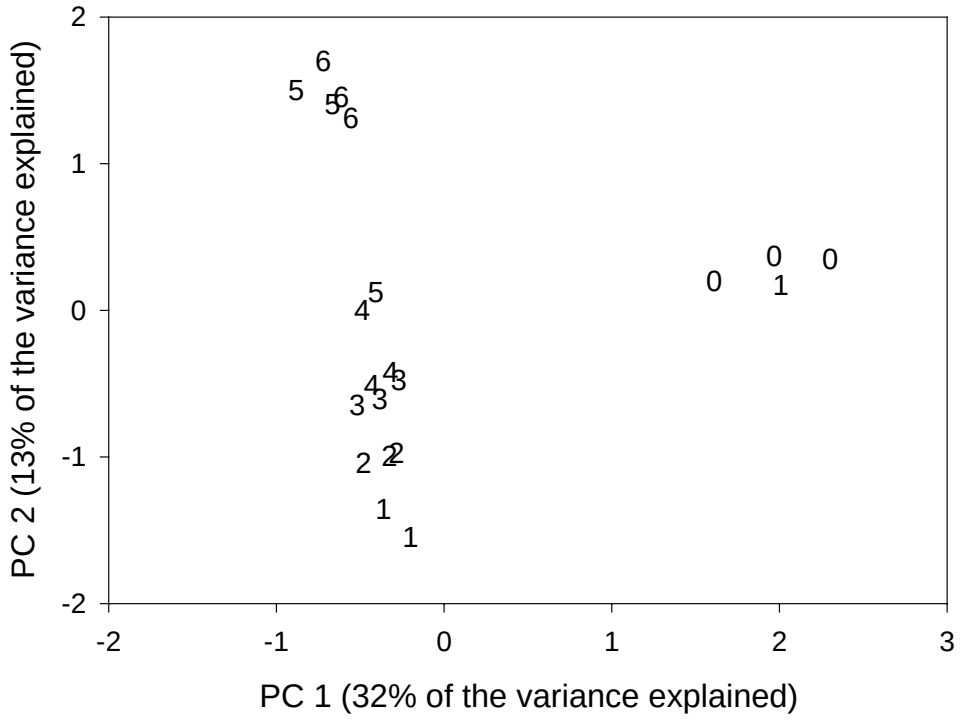

Figure 3.5. Results of a PCA of the AFLP profiles. Data are presented for each of the three replicate flasks for each treatment (each inoculum dilution) and the symbols used in the figure correspond to the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution). The percent of variance explained by each principal component is provided.

Table 3.3. Comparison of cultural and genetic procedures including the average number of fragments or average number of colony types (per dilution treatment), and the relative proportion of each that were unique to a given set of communities. "Dilution" refers to the original dilution used as the inoculum for each regrown community.

Dilution	R2A Plates		AFLP		T-RFLP			
	Avg. # of colony types	Proportion unique[a]	Avg. # of fragments	Proportion unique[a]	Avg. # of fragments (*Msp*I)	Proportion unique[a]	Avg. # of fragments (*Hha*I)	Proportion unique[a]
10^{0}	8	0.4	26	0.9	7	0.2	19	0.07
10^{-1}	4.8		24		9		15	
10^{-2}	4.7		17		11		20	
10^{-3}	5.7	0	17	0.6	10	0.2	12	0.2
10^{-4}	4		14		11		13	
10^{-5}	2.7		20		18		21	
10^{-6}	3	1.0	15	0.5	8	0.2	10	0.2

[a] Number of unique colony types (or bands) in a group of treatments divided by the total number of colony types (or bands) encountered in each treatment group. "Unique" refers to a colony type (or band) that was present in a particular group of treatments (e.g., 10^{0} and 10^{-1}) that was not present in either of the other two treatment groups.

3.3.2.6. T-RFLP

Upon digestion with the *Hha*I restriction enzyme, 43 different T-RFLP fragments were produced; on average, an individual sample contained 16 of these fragments. When the *Msp*I enzyme was used, 42 different fragments were detected; on average, a sample contained 12 of these. The number of fragments observed across the dilution gradient did not differ for either enzyme (Table 3.3). The proportion of bands unique to each of the three dilution/diversity groups (10^0 with 10^{-1}, 10^{-2} through 10^{-4}, and 10^{-5} with 10^{-6}) was also compared (Table 3.3).

Based on the principal component (Figure 3.6) and cluster analyses (results not shown) performed on the combined *Msp*I and *Hha*I datasets, two groups could be distinguished; the first group contained the communities regrown from the undiluted (10^0) through 10^{-3} inoculum, while the second group contained the lower diversity/higher dilution treatments. Again, a bootstrapping procedure (using 100 replications) was used to assess the significance of the results from the cluster analysis. The highest bootstrap value obtained (not considering bootstrap values associated with the subgrouping of the replicate flasks) was associated with the division of the communities into three groups: 10^0 through 10^{-3}, 10^{-4}, and 10^{-5} with 10^{-6}. However, the bootstrap value for these groupings was only 47, and it cannot be concluded that these three groups were significantly different. It is possible that the number of fragments being compared in the cluster analysis was insufficient, and a higher bootstrap value might be obtained if additional restriction digests (using different enzymes) were performed and the data pooled prior to statistical analyses.

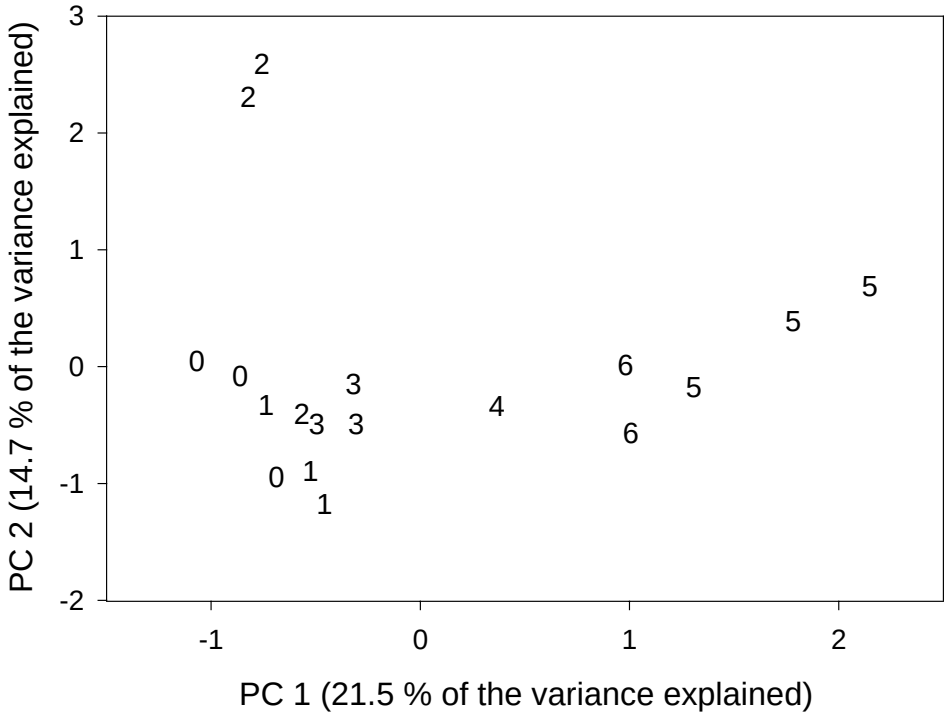

Figure 3.6. Results of a PCA of the T-RFLP profiles. Data are presented for each of the three replicate flasks for each treatment (each inoculum dilution), and the symbols used in the figure correspond to the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution). Due to experimental difficulties with the T-RFLP analysis, only one of the flasks from the 10^{-4} dilution community, and only two of the flasks from the 10^{-6} dilution community, were analyzed. The percent of variance explained by each principal component is provided.

3.4. Discussion

3.4.1. Numerical simulations

Diversity is a combination of two community attributes - richness, the number of different types of organisms in a system, and evenness, the relative distribution of individuals among the different types. The more types of organism there are, and the more nearly even their distribution, the greater the diversity of the community (Pielou, 1966). For the numerical simulations conducted in this study, richness, evenness, and overall diversity (as calculated by the Shannon-Wiener index) were compared (Figure 3.1). The results suggest that dilution of a complex microbial community does not change the overall diversity of each resultant mixture, regardless of the evenness of the original community, until the size of the community is decreased so much so that the number of individuals in the mixture approximates the original number of species. After this point, diversity must decrease with subsequent dilution (e.g., 10^{-4} and 10^{-5}) because each individual that is removed from the system always removes a species from the community. This result was anticipated for relatively even communities, but it was initially surprising to discover that diversity did not change upon dilution of the more dominant mixtures of organisms (e.g., var = 100 or var = 250 in Figure 3.1) for the early stages of the dilution series (through 10^{-3}). It had been expected that dilution of these communities would remove rare organisms from the mixture, causing overall diversity to drop; in fact, the decrease in species richness upon dilution of the highly dominant communities was substantial (Figure 3.1B). However, this decrease in species richness was accompanied by a concurrent increase in community evenness, resulting in little change in overall diversity until a 10^{-4} or 10^{-5} dilution.

These results suggest that a dilution approach may be used to create communities differing in diversity by comparing undiluted (or barely diluted) mixtures with communities regrown from very dilute inocula. This approach should be successful, regardless of the diversity and dominance relationship of the starting community; however, greater differences are to be expected for more even initial communities. Actual experimental communities regrown from diluted mixtures are not expected to exactly mimic these simulations, which only accounted for the dilution of the inoculum and not for any variance in regrowth. Synergistic and mutualistic interactions among organisms may be disrupted by the dilution procedure and, as a result, not all of the organism types carried through a dilution series to an inoculum may be able to regrow. The dilution procedure also decreases competition among organisms, and this could permit types that were not important in the original community to grow to uncharacteristically high abundances. Different growth rates among organisms may also impact the diversity of the regrown communities, changing evenness from that of the inoculum.

3.4.2. Batch culture experiments

The results of the numerical simulations were used to make specific predictions about the behavior of the diluted/regrown communities in the batch culture experiments. If the initial community was evenly distributed, community structure of the regrown mixtures would not be expected to change along the dilution gradient until the number of cells in the diluted inoculum approximated the total number of types of organisms in the original community. If the initial community was unevenly distributed, the first dilution

during inoculum preparation would have removed a large number of types of organisms (e.g., in the var=100 community, the first dilution removed 827 types out of 1000). However, this loss of richness would have been offset by a simultaneous increase in community evenness, resulting in no net change in overall inoculum diversity (as calculated using the Shannon-Wiener index). Nevertheless, regrowth of communities so different in richness may have resulted in a measurable change in diversity or community structure between the undiluted and the 10^{-1} treatments. After the initial dilution of an unevenly distributed community, evenness would be greatly increased, and so differences in subsequent dilutions (10^{-2}, 10^{-3}, 10^{-4}) were predicted to be small - until the dilution factor exceeds the original number of types of organisms in the community as described above.

3.4.2.1. Traditional microbiological methods

The regrown microbial communities were assayed using a number of traditional microbiological methods; each showed that the microbial communities regrown from the very dilute inocula (10^{-5} and 10^{-6}) were unique. Abundance (as determined by AODC and plates counts on R2A and SM) was always significantly greater in these communities. It is possible that this variation in bacterial concentration was the result of differential grazing pressure along the dilution gradient, as dilution of the inoculum could have changed the amount of predation pressure in each treatment (Juergens et al., 1997; Pernthaler et al., 1997; Sinclair and Alexander, 1989). However, given the low

concentration of ciliates and other grazers in the undiluted inoculum (direct microscopic observation, 1.5×10^2 organisms ml^{-1}), the impact of these organisms on bacterial abundance should have been small, especially when comparing dilution treatments beyond 10^{-2}.

Ecological theory predicts that when interspecific competition is decreased (as was done by dilution in this study), populations can increase substantially in abundance (Giller, 1984). In the present work, the inverse relationship observed between final community size and inoculum dilution suggests that interspecific competition may be more important than intraspecific competition in controlling total abundance. In the barely diluted communities (10^0 and 10^{-1}), where diversity, and therefore interspecific competition, was higher, community size was much smaller compared to the very dilute communities (10^{-5} and 10^{-6}), where diversity was low and interspecific competition was (presumably, due to high AODC) greater.

Percent culturability on each growth medium was much higher in the communities regrown from the high dilution inocula. Given that microbial growth on culture media recovers a limited number of organisms, due to inappropriate incubation conditions or an inability of certain types of organisms to metabolize the supplied substrates, enhanced growth by the 10^{-6} community suggests that those organisms are not as limited in their metabolic capabilities as those in the undiluted/low dilution communities. Furthermore, the ratio of sewage media to R2A counts changed along the dilution/diversity gradient; communities from the high dilution (10^{-5} and 10^{-6})

communities preferred R2A agar, while the other communities either had no preference or grew to higher abundances on the sterile sewage media; this result provides further evidence that the communities in the various dilution treatments were physiologically distinct.

The comparison of colony morphology on R2A plates showed that microbial diversity and richness of the recoverable fraction of the community decreased along the dilution gradient; the evenness of the communities increased. Based upon the dilution simulations, the greatest difference in community structure was expected between the very dilute (10^{-4} or 10^{-5}) communities and all of the others, regardless of the structure of the original community; each of the other analytical methods employed in this research showed this to be true. However, the greatest difference for the diversity on R2A plates was between the undiluted (10^{0}) and the 10^{-1} treatment. The fact that any difference in community structure was detected between these two dilution treatments suggests that the original sewage community may have had high dominance; the fact that there was no discernable change in overall diversity of colony types for the high dilution/low diversity treatments (e.g., 10^{-4}, 10^{-5}, and 10^{-6}) implies that the procedure may not be useful when making inferences about microbial community structure in low diversity situations.

One of the main criticisms of culture-based studies is that the carbon and nutrient sources found in a single culture media are not diverse enough, so only a small fraction of organisms in a sample actually form colonies on a spread plate. It has been suggested that using a large number of different media types may increase the variety of organisms recovered with a cultural approach. In this study, a sterile sewage medium was also used in an attempt to increase the number of types to compare when calculating the diversity

index. Unfortunately, the colonies that grew on the SM were quite small and generally lacked morphological distinctiveness, making a comparison among treatments impossible. Another concern with regard to the use of culture-based procedures is the difficulty in accurately and consistently identifying community members, given the fact that very similar colony morphologies can occur among taxonomically distinct groups of organisms. However, recent studies have shown that colony morphology can in fact provide an accurate basis on which to define "recoverable diversity" (Haldeman and Amy, 1993; Lebaron et al., 1998).

3.4.2.2. Genetic measures

The DNA fingerprinting approaches used in this study showed a significant difference in overall microbial community structure along the dilution gradient; in particular, analysis of both the AFLP and T-RFLP data showed that the very dilute (10^{-5} and 10^{-6}) communities were distinctly different from the communities regrown from less dilute inocula (Figures 3.5 and 3.6). AFLP also separated the undiluted community from the remaining treatments. The fact that AFLP distinguished a difference in microbial community structure between the undiluted and the 10^{-1} dilution community provides further evidence that the original microbial community (before dilution) had high dominance.

With T-RFLP, PCR is used to amplify the 16S rRNA genes directly from each community DNA sample using a pair of primers, and analysis of a community sample produces a fingerprint wherein each individual band is, theoretically, derived from a different organism type (a different ribotype). However, it is well known that T-RFLP

underestimates the species richness of a community because populations that are not numerically dominant are not represented if their template DNA comprises too small of a fraction of the total community DNA (Dunbar et al., 2000; Liu et al., 1997). Moreover, due to the conservation of restriction site positions in 16S rDNA, the resolution of T-RFLP analysis is not at the "species" level but instead reflects the distribution of higher order groups. Another limitation to the resolving power of T-RFLP is in the actual "universalness" of primer pairs, as none of the available universal primers can hybridize to all of the known eukaryotic, bacterial, or archaeal 16S rRNA genes (Brunk et al., 1996; Zheng et al., 1996). Despite these limitations, researchers commonly use T-RFLP for comparing microbial community structure and frequently interpret the "number of T-RFLP peaks" to be reflective of [minimum] community richness (Liu et al., 1997 and 1998).

In this study, the total number of T-RFLP peaks was expected to decrease along the dilution/diversity gradient, corresponding to a loss in species richness; this number instead remained essentially constant (Table 3.3). However, the identity of the T-RFLP peaks changed, and this shift is illustrated by comparing the number of unique fragments found in each dilution/diversity group (Table 3.3). The fact that several unique peaks were observed in the "low diversity" treatments (10^{-5} and 10^{-6}) was surprising. Probability suggests that the dominant organisms in the original community are the ones that should persist through the dilution procedure, and would therefore be used to inoculate these flasks. Consequently, nearly all of the organisms observed in the very dilute treatments should also have been detected in the less dilute treatments. The fact that this was not found to be the case with T-RFLP suggests either (1) a lack of

discrimative power of T-RFLP, due to the bias of the procedure toward specific and/or dominant community members (a bias that seems to change depending on the evenness of the community being analyzed) or (2) a failure of these organisms to survive in the less dilute treatments, despite their dominance in the original community and their ability to thrive in culture in the more dilute treatments.

Since the original sewage community contained 1.8×10^6 cells ml^{-1} (AODC), the most dilute community maintained in this experiment (10^{-6}) should have been inoculated with approximately 2 cells. After regrowth, diversity in this community was expected to be very low. On the R2A plates, percent culturability was high (100%) and only three colony morphologies were observed, further suggesting that diversity in these flasks was quite low. Given this information, it was surprising to find that the average number of T-RFLP fragments in the 10^{-6} treatment was so high (8 for *Msp*I and 10 for *Hha*I). It is possible that this discrepancy could have been the result of a technical error with the T-RFLP, e.g., an incomplete restriction digest, which could produce a number of differently sized T-RFLP fragments for each organism type. However, experimental controls, where the T-RFLP analysis was applied to DNA from a pure culture, were also performed and a single T-RLFP peak was generated in each case. Another possible explanation is that, because an individual organism can contain multiple, heterogeneous copies of the 16S rRNA gene (Linton et al., 1994; Ninet et al., 1996; Pettersson et al., 1994; Wang and Wang, 1997; Wang et al., 1997), each organism type could actually have been responsible for more than one T-RFLP peak. However, the extent to which such sequence deviations occur is not well studied, and it is unlikely that the detection of multiple, divergent copies of a 16S rRNA gene can account for the results presented here.

Recent work has discovered that related strains of bacteria can have the same 16S rRNA gene, but may not have the same physiological profiles or the same ecological strategies in the environment (Jaspers et al. 2001). Presumably, this additional variability is coded elsewhere on the bacterial chromosome. Consequently, analysis of a single gene may not provide as much resolution when distinguishing among communities, compared to procedures that can survey the entire genome (e.g., AFLP). With AFLP, a restriction digest is performed on a DNA sample (similar to RFLP) and then a set of primer-recognition sequences (adaptors) is used to amplify the restriction fragments using PCR (Zabeau and Vos, 1993); the primers and restriction enzymes used are not specific for a given gene or group or genes but can, theoretically, interact in numerous random places throughout a genome. AFLP is very similar in premise and application to RAPD fingerprinting, which has been used a number of times to compare microbial community structure (Franklin et al., 1999a; Franklin et al., 1999b; Wikström et al., 1999; Wikström et al., 2000; Xia et al., 1995; Yang et al., 2000).

AFLP is fundamentally different from each of the other procedures applied in this work, and from most other techniques used to compare microbial community structure, in that it is sensitive to *overall* differences between communities - including taxonomic distances between organisms. Watve and Gangal (1996) point out that most procedures would not detect a difference in diversity between one community composed of four biotypes of coliforms and another composed of one coliform, one archaebacterium, one myxobacterium, and one actinomycete - though many microbial ecologists would agree that the latter should be treated as more diverse. The ability to differentiate between such mixtures is important and it has been suggested that one way to incorporate this

additional information is to simply calculate the mean taxonomic distance between all pairs of isolates in a community as a diversity index (Rao, 1980); however the problem of isolation and taxonomic characterization of individuals remains. In this study, AFLP was used to compare overall diversity, considering richness, evenness, and taxonomic relatedness of community members without attempting to evaluate each of these elements separately.

3.4.2.3. Community-level physiological profiling

CLPP compares patterns of carbon substrate utilization among communities by evaluating the extent to which a community metabolizes each of 95 different sole carbon sources (Garland and Mills, 1991). When CLPP was applied in this study, the different dilution communities separated into two distinct groups (10^{-5} and 10^{-6} were unique (Figure 3.3)). These results are important as they demonstrate that there were phenotypic differences among the regrown communities, as well as the genetic differences already described. This means that the dilution process not only changed the identity of the organisms in the communities, as revealed by the genetic analyses, but also changed the communities' overall metabolic capabilities – the most diverse (undiluted) community did not have the same functional potential as the low diversity (10^{-6}) community. The fact that the genetic and the phenotypic measures gave similar results in this study is also meaningful as the correlation between the two suggests that genetic differences among communities actually have the potential to manifest as differences in function.

Dilution/extinction analysis of CLPP was used to compare relative structural diversity of the different regrown communities. This procedure uses dilution to extinction of a heterotrophic microbial community to evaluate the rate of character loss from the mixture; assuming the rate of character loss is somehow proportional to the diversity of the original community, the relative diversity of the sample can be estimated (Garland and Lehman, 1999). In this study, dilution/extinction analysis showed no significant change in the maximum functional richness (R_{max}) of each community along the diversity gradient. The other regression parameter, K_I, the half-maximum richness, describes the rate at which functional characters can be diluted out of a mixed community and has been used to assess relative structural diversity in a number of different experimental systems; higher K_I correlates with a higher diversity and also with increased niche specialization (Garland and Lehman, 1999). In this study, K_I decreased along the dilution/diversity gradient confirming that the communities regrown from the less dilute inocula were more diverse. The community regrown from the undiluted inoculum was able to perform a wide variety of functions but lost this ability rapidly upon dilution; this suggests a community composed primarily of specialists. The low diversity community, regrown from the 10^{-6} dilution inocula, had a much lower K_I; this increased conservation of function among the individuals in the group may suggest a community of generalists. The results of the percent culturability calculations also showed the high dilution/low diversity communities to be more generalized in their metabolic capabilities.

Often times, when researchers are advocating the use of molecular techniques over culture-based procedures, the reason presented is that culture-based analyses are too biased toward certain groups of organisms. As such, they underestimate the total richness of a community in an inconsistent and unpredictable manner. In this research, 23 colony types were observed on R2A agar (across all treatments), and only 42 unique T-RFLPs were encountered. Certainly, the actual total number of organism types in the original sample was much greater. Considering that each of the analytical methods employed in this study showed a clear change in overall community structure between the 10^{-4} and 10^{-5} dilution treatments (Table 3.4), the numerical simulations would then suggest that there were between 1000 and 10,000 types of organisms in the original sewage community. This value is consistent with results of Torsvik et al. who found that a gram of soil can contained between 4,000 (Torsvik et al., 1990) and 10,000 different bacterial types (Torsvik et al., 1993). In the present study, a more precise estimate of the number of types in the originial community might have been obtained if a different dilution scheme had been used (e.g., intervals smaller than 10-fold).

In this study, significant differences in community structure were detected using genetic (AFLP and T-RFLP), physiological (CLPP), and culture-based (colony morphology on R2A agar) measures. Along with this difference in community structure, differences in community size (AODC), composition (ratio of sewage medium counts to R2A counts, monitoring of each colony morphology across the treatments), and metabolic redundancy (generalist versus specialist) were observed, suggesting that differences in structure/diversity of communities maintained in the same environment can

manifest as differences in community organization and function. Though differences in microbial community structure were detected with every measure employed, each procedure had different methodological limitations that should be recognized when that technique is applied. The results of the experimental incubation demonstrated that the dilution/regrowth approach may be a useful way of generating communities differing in diversity (richness and evenness) and varying in overall community structure. Moreover, the procedure may be a useful was of analyzing communities – in this study, a great deal of information was gained about the richness and distribution of the original sewage community from analysis of the regrown communities.

Table 3.4. Summary of the groupings obtained using each technique. Different letters represent significantly different communities, determined as described in the text. "Dilution" refers to the original dilution used as the inoculum for the regrown communities.

Dilution	AODC	R2A counts	R2A: % culturability	Diversity on R2A plates†	SM counts	Ratio of SM to R2A counts†	CLPP	Diln./ Extn. of CLPP†	AFLP	T-RFLP
10^{0}	a	a	a	a	a	a	a	a	a	a
10^{-1}	a	a	a	b	a	a	a	-	b	a
10^{-2}	a	a	a	b	a	a	a	b	b	a
10^{-3}	a	a	ab	b	ab	a	ab	-	b	a
10^{-4}	ab	a	ab	b	ab	a	ab	b	b	ab
10^{-5}	b	ab	b	b	ab	b	b	-	c	b
10^{-6}	c	b	c	b	b	b	c	c	c	b

† For these data, statistical analyses were not performed to assess significance levels; instead the different groupings outlined above were determined by visual interpretation of Figure 3.2 ("Diversity on R2A plates") and Figure 3.4 ("Diln./Extn. of CLPP"). For the ratio of SM to R2A counts, different groups were defined as having a ratio greater than 1 (a) or less than 1 (b).

Chapter 4.

Structural and functional responses of a sewage microbial community to dilution-induced reductions in diversity.

Abstract

The purpose of the research presented here was to examine the relationship between functional redundancy and microbial community structure/diversity using culture experiments to ensure constant environmental conditions. Serial dilutions of a sewage microbial community were prepared, used to inoculate sterile sewage, and maintained in batch culture. Probability suggests that dilution of the initial community should remove rare organism types, creating mixtures of cells differing in diversity. Regrowth of the diluted mixtures generated communities similar in abundance but differing in community structure (determined using two DNA fingerprinting techniques) and relative diversity (evaluated using community-level physiological profiles (CLPP)). The *in situ* function of each regrown community was examined by monitoring the short-term uptake of five different ^{14}C labeled compounds (glucose, acetate, citrate, palmitic acid, and an amino acid mixture). No significant differences were detected between treatments in either the rate of uptake of a substrate or the efficiency with which each community assimilated each compound. However, changes in community-level glucose and acetate metabolism were observed, and correlated with specific changes in community structure. This suggests that a significant loss of function might have been observed if additional treatments, with lower levels of diversity, had been maintained. Nonetheless, the fact that the activity of the original community was the same as that of a community regrown from a 10^{-6} dilution indicates that functional redundancy was quite high in this system and may contribute to ecosystem stability. For each organism type eliminated during the dilution process, at least one of the remaining organism types was able to provide the same function at the same level as the lost type.

4.1. Introduction

Over the past 50 years, there has been intense research and debate in the field of ecology about the significance of biodiversity in ecosystem function. Many ecological theories, developed from the study of plant and animal communities, propose that there is a relationship between diversity and important ecosystem processes such as resource partitioning, succession, productivity, community function, and ecosystem stability (Elton, 1958; Hutchinson, 1959; MacArthur, 1957; Odum, 1983; Pielou, 1975). Although there is research to support these relationships in some systems (Naeem and Li, 1997; Naeem et al., 1994; Tilman, 1996; Tilman and Downing, 1994; Tilman et al., 1996), there is considerable debate over what aspects of a community, and what particular features of an ecosystem, should be compared (Chapin et al., 1997; Grime, 1997; Hooper and Vitousek, 1997; Tilman et al., 1997; Wardle and Giller, 1996). These theories have been tentatively applied to the study of bacterial communities, but methodological limitations have made rigorous hypothesis testing especially difficult. Because of the limitations of culture methods, and the extreme diversity and abundance of microbial communities, it is not reasonable to use ecological approaches that rely on determining the distribution of different types of organisms as a means of testing hypotheses about microbial community dynamics. The use of molecular techniques and community-level analyses has greatly increased our ability to monitor the distribution of bacteria in the environment; however, it is still not possible to completely categorize the constituents of a community, or to thoroughly understand the functional abilities of each individual organism type.

In addition to the taxonomic and methodological difficulties associated with monitoring the hyperdiversity of microbial communities, there are several conceptual issues, unique to the field of microbial ecology, that suggest that the use of diversity as a indicator of ecosystem performance may not be appropriate in these systems. For example, Tate (1997) questioned whether it is even useful to distinguish between "higher" and "lower" diversity in natural microbial communities, considering their tremendous richness (even for "low" diversity communities) and the fact that they may contain numerous dormant populations. The physiological versatility of many microorganisms presents another difficulty in determining the nature of any relationship between microbial community structure and function. A number of studies have shown that the broad-scale functional ability of a microbial community is often not controlled by organism diversity (Atlas et al., 1991; Degens, 1998; Fernandez et al., 1999; Klein et al., 1986), and it has been suggested that redundancy of function may be much more important for understanding the stability of microbial communities, and of the ecosystem functions they perform, than traditional diversity measures (Beare et al., 1995; Briones and Raskin, ; Finlay et al., 1997; Kennedy and Smith, 1995; White, 1995; Zak et al., 1994).

The following calculation illustrates the potential for functional redundancy in microbial communities. First, consider the distribution of genetic determinants for *E. coli*. Current estimates indicate that approximately 1/3rd of *E. coli* genes code for products that are used in cellular maintenance activities (e.g., cellular structure, transport functions, RNAs, and protein synthesis), and approximately 2,500 genes are available for

metabolic processes (1,300 already identified and 1,200 hypothesized) (Riley, 1999). Using this value to represent the average number of unique metabolic enzymes that a bacterium is *capable* of producing, it is possible to calculate the total number of genes (enzymes) available for ecosystem function in a microbial community. Using Torsvik's estimate of 10,000 different bacterial types per 30 g soil sample (Torsvik et al., 1998; Torsvik et al., 1996), there may be up to 2.5×10^7 enzymes (2,500 genes × 10,000 types) available for metabolic processing and ecosystem function in this small mass of soil. The number of enzymes required to perform any given function may vary greatly, but even if 50 different types of enzymes are required for each unique function, a community could still contain enough genetic material to code for 5×10^5 different processes (2.5×10^7 enzymes per community / 50 enzymes per function). Clearly, complex microbial communities have the potential for tremendous metabolic redundancy and may contain multiple types of organisms (species) capable of performing each function in the environment.

The hypothesized relationship between functional redundancy and stability within ecosystems is conceptually similar to a cybernetic control mechanism called "congeneric homotaxis" (Hill and Wiegert, 1980). In this case, stability is conferred on a system because multiple genera are capable of carrying out a given function in the ecosystem, presumably across a wide range of environmental conditions. If one type of organism is eliminated from the system, or ceases to function for any reason, another set of "ecologically equivalent" (Gitay et al., 1996) organisms already present in the system will instead provide the function. This allows maintenance of the system's functional

ability at or near the level prior to the loss of the first set of organisms, and acts as insurance again the loss of function from a community.

Although it is a potentially important ecosystem performance parameter, the functional redundancy of microbial communities has not been well studied (Briones and Raskin, 2003). Most recent work has focused on manipulating microbial diversity in the lab, while monitoring the associated change in function (Griffiths et al., 2000; Griffiths et al., 2001; McGrady-Steed et al., 1997; Mikola and Setala, 1998; Naeem and Li, 1997; Salonius, 1981). Another set of studies has focused on field comparisons, where differences in microbial diversity were predicted/inferred based on a historical knowledge of the communities' development (e.g., along a soil reclamation gradient (Yin et al., 2000), in association with different agricultural land-uses (Degens et al., 2001), or in response to a metal contamination (Rasmussen and Sorensen, 2001; Wenderoth and Reber, 1999)). Some of these studies have defined functional redundancy as simply the presence of multiple species that are *capable* of performing each function (i.e., redundancy of *functional potential*), and have attempted to quantify the extent of this redundancy (e.g., how many species may be found within each functional group or guild (Yin et al., 2000)). However, studies that consider how *in situ* function is affected by changes in community diversity are also needed, as there are several reasons why overall ecosystem functioning may not be maintained even in a community that is redundant with respect to each individual function. For example, though multiple populations may be capable of performing a function, they may not all perform it with the same efficiency, or they may not generate the same metabolic by-products. Similarly, a "replacement"

species may not have the same growth rate or competitive ability as the original community member. Changes such as this could influence the activity of other populations in the community, and indirectly cause a change in overall ecosystem function - despite the fact that the original function of interest has been maintained (Chapin et al., 1997). More research is needed to determine whether the presence of multiple taxa/species, with overlapping functional abilities, actually results in functional stability.

The purpose of the research presented here was to examine the relationship between functional redundancy and microbial community structure/diversity using culture experiments to ensure constant environmental conditions. Serial dilutions of a sewage microbial community were prepared, used to inoculate sterile sewage, and maintained in batch culture. Probability suggests that dilution of a relatively diverse community will remove rare organism types, creating mixtures of cells differing in species richness (Franklin et al., 2001). Regrowth of the diluted mixtures can then be used to produce mixed cultures with roughly the same biomass, but differing in overall diversity. Previous work in our laboratory has demonstrated that this approach is an effective means of generating communities differing in structure as determined using genetic, physiological, and culture-based techniques (Franklin et al., 2001; Garland et al., 1999). In the present study, several different methods were used to characterize the microbial communities, and the results indicate that the dilution/regrowth procedure did produce microbial communities that differed in overall structure and diversity. The *in situ* function (short-term uptake of five different ^{14}C labeled compounds) of each

community was measured, and there were no significant differences between treatments in either the rate of uptake of a substrate or the assimilation efficiency for any of the compounds studied. The fact that function was maintained suggests that functional redundancy was quite high within the microbial consortium, and conferred some stability on the community – at least for the functions we examined.

4.2. Materials and methods

4.2.1. Microcosm setup

A single large sample (approximately 20 L) of sewage was collected from the aeration basin of the Rivanna Wastewater Treatment Plant in Charlottesville, Virginia. After heat sterilization (autoclaving for 2 h at 120°C and 15 psi), the sample was allowed to settle for 72 h at 4°C. The clarified supernatant liquid was then used as the growth medium for the flask experiments. Cultures were prepared by adding 60 ml of autoclaved sewage supernatant to a 125-ml Erlenmeyer flask, capped with a sterile foam plug.

At the start of the experiment, a fresh sample of sewage was collected from the same aeration basin. From this sample, decimal dilutions were prepared, using sterile sewage as the diluent, and each of these dilutions was used as an inoculum in the flask experiments. Seven treatments were established by adding 1 ml of sewage ("10^0 treatment") or diluted sewage (10^{-1} through 10^{-6}) to each flask; three flasks were inoculated for each treatment. After inoculation, flasks were placed on a shaker table.

(150 rpm) and incubated at 23°C. Each day, 20 ml of fluid was removed from each microcosm and replaced with 20 ml of sterile sewage. The cultures were maintained in this fashion for 9 days (i.e., 3 retention times).

At the end of the experiment, several attributes of the regrown communities were measured including: community size (total and cultural counts), relative structural diversity (using dilution-extinction analysis of community-level physiological profiling (CLPP) (Garland and Lehman, 1999)), overall similarity in community structure (using Amplified Fragment Length Polymorphism (AFLP) DNA fingerprinting (Franklin et al., 2001; Zabeau and Vos, 1993)), community similarity based on the distribution of 16S rRNA genes (using Terminal Restriction Fragment Length Polymorphism (T-RFLP) DNA fingerprinting (Liu et al., 1997)), and short-term uptake of several ^{14}C labeled substrates. Because of the amount of laboratory effort required, final analysis of the communities took place over a two-day time period. On the first day (day 8), Acridine Orange direct counts (AODC (Hobbie et al., 1977)) were performed, the heterotrophic uptake of three different ^{14}C labeled substrates (glucose, an amino acid mixture, and citric acid) was measured, and a sample was preserved for molecular genetic analyses. On the second day (day 9), samples were again collected for AODC and genetic analyses, as well as for cultural counts on R2A agar (spread plates incubated for 48 h at 23°C). In addition, the heterotrophic uptake of two other ^{14}C labeled substrates (acetate and palmitic acid) was measured, and dilution-extinction analysis of CLPP was performed.

4.2.2. Determination of relative structural diversity

Dilution-extinction analysis of CLPP was performed on a subset of the regrown communities (two flasks from each treatment) to determine the relationship between cell density (I) and functional richness (number of positive wells (R)) for each treatment (Garland and Lehman, 1999). This information was then used to make inferences about the relative structural diversity of each set of communities. At the end of the experiment, serial dilutions of the regrown microbial communities were prepared (10^{-1} through 10^{-8}), and inoculated into Biolog GN2 microplates (Biolog, Inc., Hayward, CA). Plates were incubated at 23°C for 6 days, at which time the absorbance (590 nm) in each well of each plate was measured using a Labsystems Multiskan RC plate reader. A positive response was defined as any value greater than 0.25 absorbance units, after correction for the control well.

Data for each treatment were pooled and plots were made of the number of positive responses (R) in each plate versus the concentration of cells in each well (I) as determined using R2A counts. A hyperbolic model, $R = (R_{max} \times I) / (K_I + I)$, was then fit to the data; non-linear regressions were performed using SigmaPlot (Version 5.0), and the parameters R_{max}, which equals the maximum (asymptotic level) of R, and K_I, which is the value of I when R is 1/2 of R_{max}, were determined.

4.2.3. Molecular genetic analyses

4.2.3.1. DNA extraction and quantification

Samples (20 ml) were collected from each of the three replicate flasks for each treatment at the end of the experiment (day 9), and from one flask of each treatment on day 8. The suspended microbial community was concentrated from this solution by centrifugation (23,000 × g for 20 min), and the resultant cell pellet was resuspended in 200 µl of phosphate-buffered saline (PBS, pH 7.4) and stored at -20°C. Whole-community DNA was later extracted using the High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's instructions for bacterial cultures. DNA concentration was determined using the PicoGreen dsDNA quantification reagent (Molecular Probes, Eugene, OR).

4.2.3.2. Comparison of overall community structure using AFLP

AFLP analysis was completed using the primers and protocols described in Franklin et al. (2001) and the Perkin Elmer Microbial Fingerprinting Kit (PE Applied Biosystems, Foster City, CA). The AFLP amplification products were resolved using an ABI Prism 310 Genetic Analyzer following the manufacturer's instructions. Data were analyzed using the Genotyper software (PE Applied Biosystems), and the presence or absence of each fragment/peak in each sample was coded as 1 or 0. This type of data matrix was prepared for each primer pair, and the information from the three matrices was pooled into a single large data set. This resulted in a total of 114 bands, all of which were polymorphic.

The Jaccard coefficient was used to calculate the relative similarity between each sample pair (Sneath and Sokal, 1973), and a Mantel test (Mantel, 1967; Sokal, 1979) was used to evaluate whether overall microbial community structure was significantly different among the treatments. In general, a Mantel test determines the amount of correlation between two matrices, and a permutation procedure is used to assess the significance of this correlation. In this application, the two matrices being compared were (1) the observed similarity matrix calculated from the AFLP data, and (2) a conceptual model matrix that defined group identity (dilution treatment). Data were analyzed using the Mantel-Struct program (Miller, 1999) to test the null hypothesis that there was no difference in within- and between-group genetic similarities of the communities. A Monte Carlo procedure (using 5000 permutations) was employed to assess the significance of the test statistic, and a Bonferonni type correction was made to adjust the α level, depending on the number of comparisons.

A principal components analysis (PCA) was performed on the original pooled data matrix (SPSS 10.0), and plots of the first two principal components were made. PCA of binary data positions objects in multidimensional space at distances that are the square roots of complements of simple matching coefficients (Gower, 1966; Legendre and Legendre, 1998). The application of PCA in this study was solely to aid in visualization of the relationships among the samples and not in statistical evaluation; statistical significance was established via the Mantel tests.

4.2.3.3. Comparing community composition using T-RFLP

T-RFLP was performed as described in Liu et al. (1997), with slight modification. The eubacterial primers 27 Forward (5' AGA GTT TGA TCC TGG CTC AG 3' (fluorescently labeled with 6-FAM (5[6]-carboxy-fluorescein))) and 1492 Reverse (5' GGT TAC CTT GTT ACG ACT T3') (Operon Technologies Alameda, CA) were used. The PCR reaction mixture included: 1X PCR buffer, 200 mM of each dNTP, 2.0 mM $MgCl_2$, 1.0 μM of each primer, 0.4 μg μl^{-1} bovine serum albumin (BSA), and 1.25 units of Ampli Taq DNA polymerase (PE Applied Biosystems, Foster City, CA) per 50-μl reaction. The thermal cycling conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of: 94°C for 0.5 min, 58°C for 1 min, 72°C for 2 min, with a final elongation at 72°C for 10 min. PCR products were purified using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and eluated in a final volume of 50 μl. Separate portions (10 μl) of the purified PCR product were then digested with either the *HhaI* and *RsaI* restriction enzymes (New England Biolabs, Beverly, MA).

Data were collected using an ABI Prism 310 Genetic Analyzer. The presence or absence of each terminal restriction fragment (T-RF) in each sample was determined and recorded as a matrix of 1's and 0's. The data from the two restriction digests were pooled into a single large dataset, and analyzed using a Mantel test and a PCA as described for AFLP.

4.2.4. Short-term heterotrophic uptake assay

The following substrates, labeled with ^{14}C, were used: (a) D-[$^{14}C(U)$] glucose (specific activity: 340 mCi $mmol^{-1}$), (b) [1, 5-^{14}C] citric acid (specific activity: 83.8 mCi $mmol^{-1}$), (c) [2-^{14}C] acetate (sodium salt, specific activity: 51.2 mCi $mmol^{-1}$), (d) [1-^{14}C] palmitic acid (specific activity: 52 mCi $mmol^{-1}$), and (e) L-amino acid mixture (specific activity: 2.23 μCi μg C^{-1}, which is equivalent to 334 μCi μmol^{-1} assuming an average molecular weight of amino acids as 150 g mol^{-1}). Glucose and palmitic acid were obtained from American Radiolabeled Chemicals, Inc (St. Louis, MO), and all other substrates were obtained from NEN Life Sciences Products (Boston, MA). An additional set of experiments was performed using a sixth substrate, radiolabeled benzene, but the results are not reported here as it was later determined that the benzene stock solution had been contaminated.

Two of the replicate flasks from each treatment were randomly selected for analysis of heterotrophic activity (King, 2002; Wright and Burnison, 1979). From each flask, 5 ml of the culture were removed and mixed with 5 ml of sterile sewage and 0.1 μCi of radiolabeled substrate in a 25-ml Erlenmeyer flask. At the end of a 2-hr incubation period, the amount of ^{14}C labeled CO_2 produced in each flask was determined, and represented the portion of substrate taken up by the community and respired (mineralization). The amount of substrate taken up and accumulated into biomass (assimilation) was estimated by filtering the sewage through a 0.4 μm filter, to trap the microbial cells, and then quantifying the amount of radiolabel associated with the filter. Radioactivity was measured using a Beckman LS 6500 liquid scintillation counter. Total

uptake was calculated as the sum of mineralization and assimilation, and standardized to a per cell value using the AODC; assimilation efficiency was calculated as the portion of the total label uptake incorporated into biomass. For each substrate, each of these parameters was compared across treatments using an ANOVA.

4.3. Results

4.3.1. Community size

An ANOVA of the AODC data (Figure 4.1A) showed no significant differences in the total concentration of cells across the various dilution/diversity treatments for either day 8 (d.f. = 13, F = 0.798, p = 0.606) or day 9 (d.f. = 20, F = 1.397, p = 0.283) of the experiment, and there were no significant differences between days (d.f. = 27, F = 0.498, p = 0.492). The average concentration of cells based upon these two sets of measurements was 3.5×10^8 cells ml^{-1} ($\pm 3.3 \times 10^7$ (SEM)), and the concentration of cells in the original undiluted inoculum (at the start of the experiment) was 2.2×10^8 cells ml^{-1}.

A second ANOVA demonstrated that the number of colonies able to grow on R2A agar (Figure 4.1B) was significantly different across treatments (d.f. = 18, F = 4.39, p = 0.014). Post-hoc pairwise comparisons (Tukey HSD) indicated that the community regrown from the 10^{-5} dilution inocula was significantly different from all of the others. The average concentration for the 10^{-5} treatment was 1.9×10^8 CFU ml^{-1} (colony forming units), and the average for all of the other treatments combined was only 2.4×10^7 CFU ml^{-1} ($\pm 8.1 \times 10^6$ (SEM)). The concentration of cells in the original inoculum that were capable of growing on R2A agar was 2.3×10^6 CFU ml^{-1}.

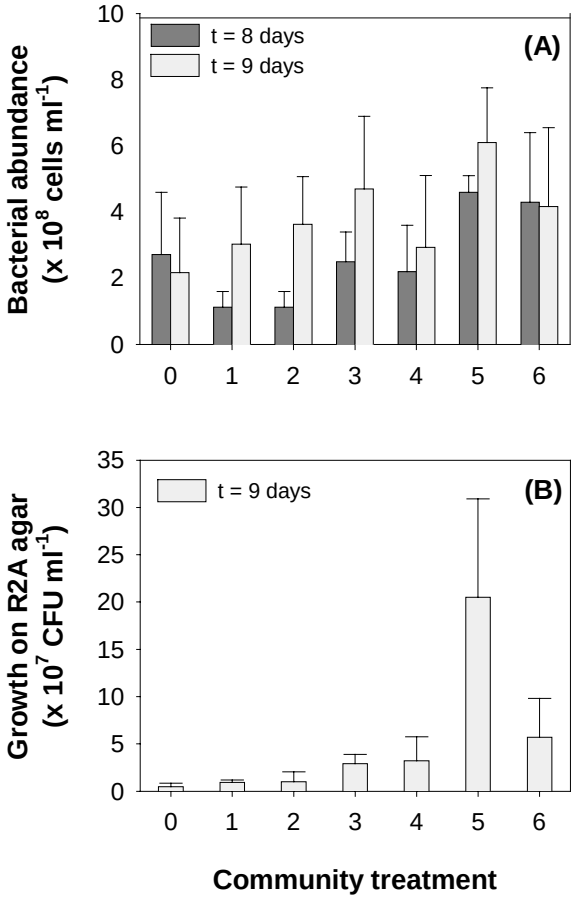

Figure 4.1. Bacterial abundance (mean ± 1 SD) as determined by AODC (A) and cultural counts on R2A agar (B). The X-axis in each of these graphs represents the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution treatment).

4.3.2. Relative structural diversity

Dilution-extinction analysis of functional characters in the CLPP assays was used to make inferences about the relative structural diversity of each of the regrown microbial communities. Two flasks from each treatment were analyzed, the data pooled, and plots were made of the functional richness (R) versus inoculum concentration (I), as described in the Methods section. The data were fit with a rectangular hyperbola to estimate the parameters R_{max} and K_I for each treatment (Figure 4.2); the multiple correlation coefficients (R^2) for these fits were quite high (10^0, 10^{-2}, and 10^{-3} treatments: 0.94, 10^{-4}: 0.90, 10^{-5}: 0.98, and 10^{-6}: 0.80), and all p values < 0.0001. Because of an error made when inoculating the plates from the 10^{-1} treatment, no values are available for that set of communities.

R_{max}, referred to as maximum functional richness by Garland and Lehman (1999), is the estimated maximum number of tests that would be positive for a community. This value ranged from 82 to 92, and did not change significantly along the dilution/diversity gradient (Figure 4.2 A). However, K_I (Figure 4.2 B), the cell density at which R (functional richness) = 1/2 (R_{max}), decreased in the communities that were predicted to have lower diversity based upon extent of dilution.

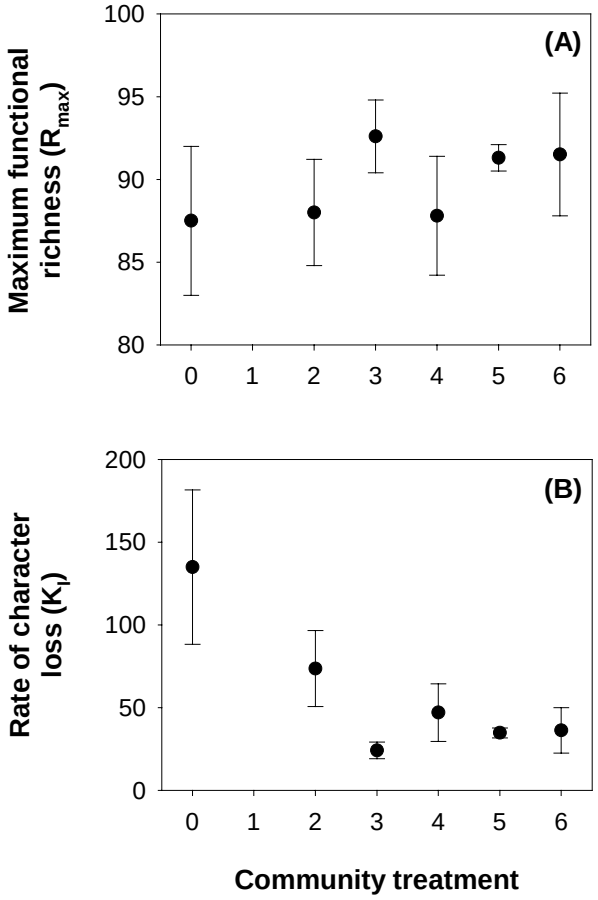

Figure 4.2. Mean parameter estimates (± 1 SD) obtained from the dilution-extinction analysis of CLPP: maximum function richness, R_{max} (A) and rate of character loss, K_I (B). The X-axis in each of these graphs represents the negative exponent of the dilution factor used to create the original inoculum (e.g. "4" corresponds to a 10^{-4} dilution treatment).

4.3.3. Overall community genetic structure (AFLP)

Principal components analysis of the AFLP data (Figure 4.3 A) separated the communities into four main groups based upon overall genetic composition: undiluted (10^0), 10^{-1}, 10^{-2} through 10^{-5}, and 10^{-6}. The four data points shown for each treatment represent a single sample from each of the three flasks on day 9, and a single sample from one randomly selected flask on day 8. The four data points always cluster as a group, indicating that the communities were as similar over time (day 8 and day 9) as they were among replicate flasks. A Mantel test was performed on the AFLP data to test whether the communities from the different dilution/diversity treatments were significantly different. After applying a Bonferonni procedure to correct the significance level for multiple comparisons (initial $\alpha = 0.05$), three significantly different subsets were established: undiluted (10^0) with 10^{-1}, 10^{-2} through 10^{-5}, and 10^{-6}.

4.3.4. T-RFLP analysis of dominant community members

In the T-RFLP analysis, 38 T-RF were observed across all treatments (pooled results for two separate enzymes); only 7 of these T-RFs were common to all of the communities, 5 were found only in the low-dilution treatments (10^0 - 10^{-4}), and 16 were found only in high-dilution treatments (10^{-5} and/or 10^{-6}). The number of T-RFs found for each treatment did not change significantly or consistently across the dilution series, except that the high-dilution treatments (10^{-5} and 10^{-6}) generally contained the most T-RF. As with the AFLP data, there were no significant differences in the samples collected on separate days.

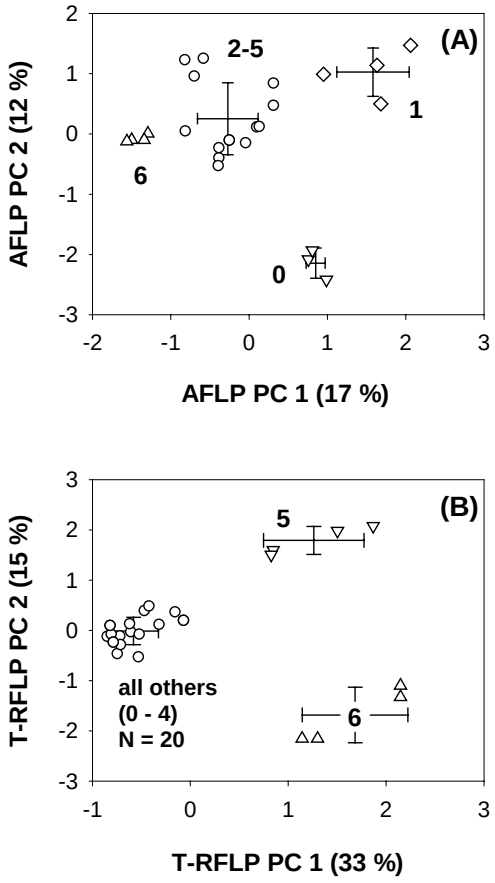

Figure 4.3. PCA of the AFLP (A) and T-RFLP (B) DNA fingerprinting data (includes mean ± 1 SD). Different symbols are used to distinguish significantly different groups, and different sets of error bars are associated with each set of symbols. Treatments are identified by the negative exponent of the dilution factor used to create the original inoculum (e.g. "4" corresponds to a 10^{-4} dilution treatment). The percent of variance explained by each PC is provided. For AFLP, no data were collected from one of the communities regrown from the undiluted inoculum and for two of samples from the 10^{-2} dilution.

The PCA of the pooled T-RFLP profiles (Figure 4.3 B) showed that the communities from the high-dilution treatments (10^{-5} and 10^{-6}) were distinct from all of the others, and somewhat different from one another. A Mantel test was performed as described for AFLP, and the communities from the low-dilution treatments (undiluted (10^{0}) through 10^{-4}) were found to be distinct from both the 10^{-5} and the 10^{-6} dilution treatments. The two high-dilution treatments (10^{-5} and 10^{-6}) were nearly significantly different after correction for multiple comparisons ($p = 0.03$).

4.3.5. Short-term heterotrophic uptake assays

For each substrate, total uptake (Figure 4.4) and assimilation efficiency (Figure 4.5) were calculated for each treatment and expressed per AODC cell. No statistically significant differences were detected between any of the treatments for any of the substrates (ANOVA). For palmitic acid, assimilation could not be measured because the substrate adhered to the cells and/or filters, so instead respiration (normalized per cell) was compared across treatments using an ANOVA (Figure 4.6). As with the other substrates, no significant differences were observed. Because the assimilation of palmitic acid could not be measured, the efficiency was not calculated.

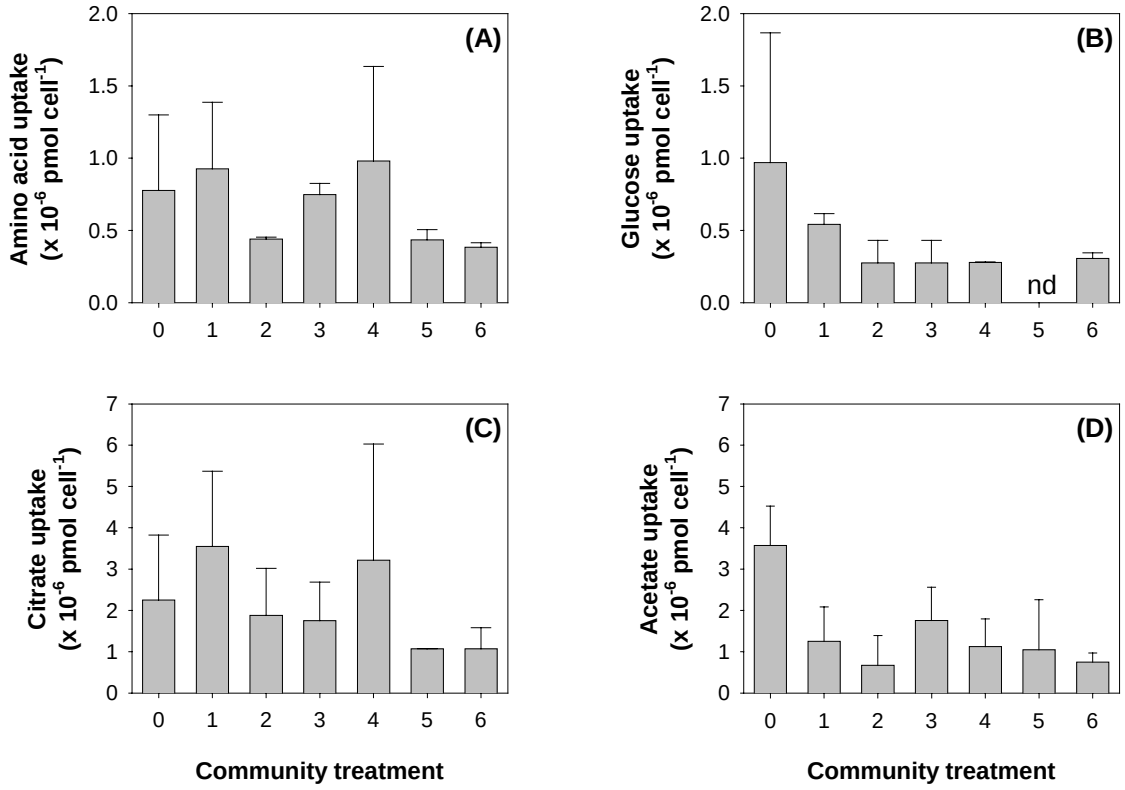

Figure 4.4. Total uptake of each ^{14}C-labeled substrate per AODC cell during the two-hour incubation period (mean ± 1 SD) for the amino acid mixture (A), glucose (B), citrate (C), and acetate (D). The X-axis in each of these graphs represents the negative exponent of the dilution factor used to create the original inoculum (e.g. "4" corresponds to a 10^{-4} dilution treatment). 'ND' means 'not determined.'

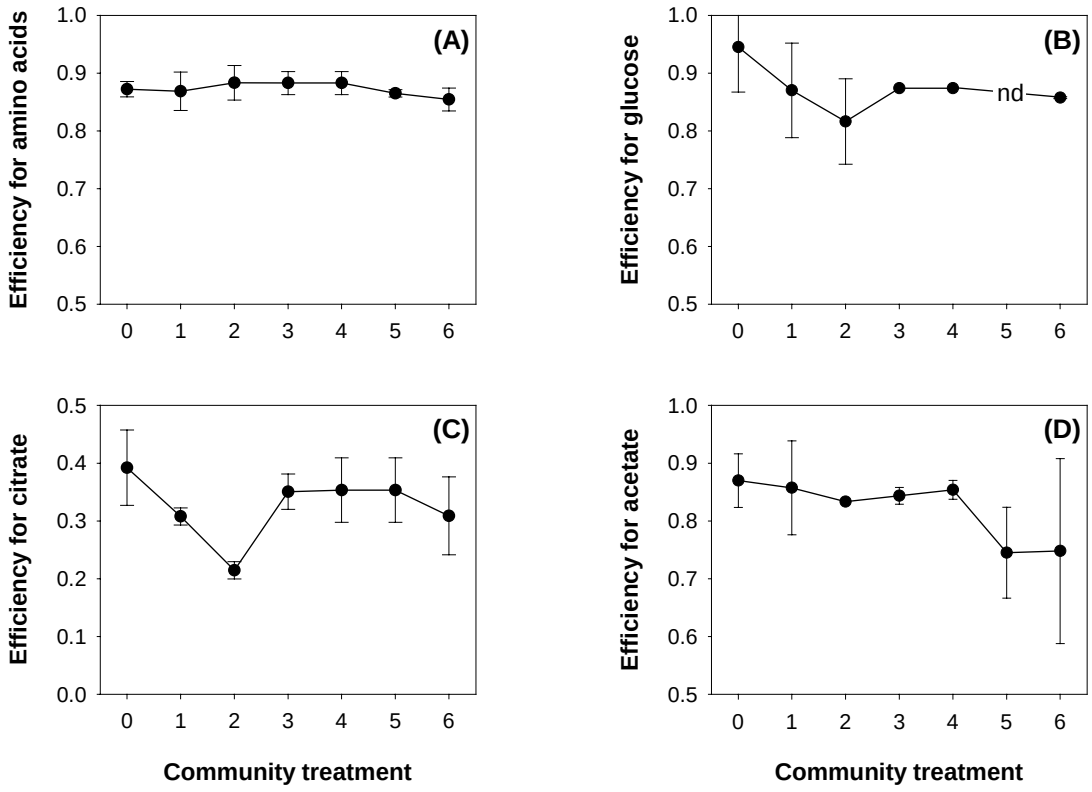

Figure 4.5. Assimilation efficiency of each ^{14}C-labeled substrate during the two-hour incubation period (mean ± 1 SD) for the amino acid mixture (A), glucose (B), citrate (C), and acetate (D). The X-axis in each of these graphs represents the negative exponent of the dilution factor used to create the original inoculum (e.g. "4" corresponds to a 10^{-4} dilution treatment). 'ND' means 'not determined.'

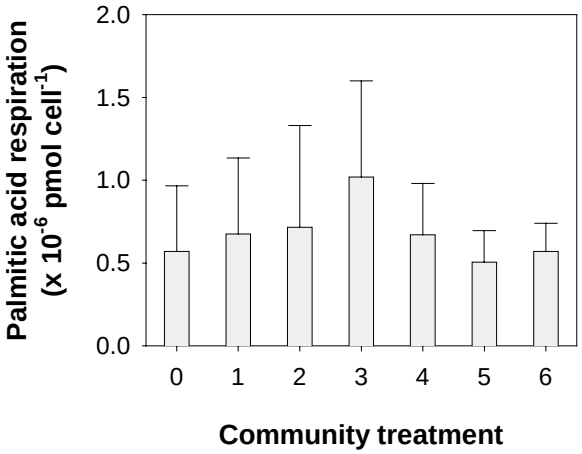

Figure 4.6. Respiration of ^{14}C-labeled palmitic acid per AODC cell during the two-hour incubation period (mean ± 1 SD). The X-axis on this graph represents the negative exponent of the dilution factor used to create the original inoculum (e.g. "4" corresponds to a 10^{-4} dilution treatment).

4.4. Discussion

The dilution/regrowth approach described here has been used a number of times as a means of manipulating diversity in microbial systems (Franklin et al., 2001; Garland and Lehman, 1999; Garland et al., 1999; Griffiths et al., 2001; Morales et al., 1996; Salonius, 1981). These studies have shown that regrown communities may differ in overall community structure, relative diversity, functional potential, and metabolic redundancy, and that more diverse communities, those regrown from less dilute mixtures, may be more stable (e.g., better able to withstand an invasion attempt (Garland et al., 1999; Morales et al., 1996)). The purpose of the present study was to use the dilution/regrowth procedure to generate a set of communities, similar in composition but systematically differing in diversity, and to monitor any associated changes in community function. In addition to monitoring the short-term *in situ* uptake of several substrates, the communities were analyzed to confirm that the procedure actually generated communities with different structure and diversity.

Microbial community structure was analyzed using three different techniques: (1) AFLP fingerprinting, to compare overall genetic structure between each pair of communities, (2) T-RFLP, to compare the dominant organism types in each treatment based on variability in the 16S rRNA gene, and (3) dilution-extinction of CLPP, which allowed ranking of the communities based upon relative diversity using a phenotypic assay. All three approaches confirmed that the microbial communities grown from more dilute inocula (10^{-5} and 10^{-6}) were less diverse, and had significantly different community structure, compared to the communities grown from the undiluted inocula (10^{0}). Despite

the fact that microbial community structure and diversity changed along the dilution gradient, there were no significant differences in either the uptake rate (how rapidly the communities were able to process a given substrate) or the efficiency of the communities (how they partition the substrate between biomass and loss to respiration) for glucose, acetate, citrate, or the amino acids mixture (Figures 4.4 and 4.5). Community respiration of palmitic acid also did not vary significantly across treatments (Figure 4.6).

All of the communities in this study were incubated in the same environment, supplied with the same growth medium, and inoculated with a similar group of organisms. Therefore, it was not necessarily surprising to discover that they performed several functions to the same extent, given how tightly coupled microbial activities are to physical and chemical surroundings. The fact that the activity of the communities regrown from the very dilute inocula (10^{-6}) could be the same as the communities regrown from the undiluted (10^{0}) one suggests, as the calculations in the Introduction did, that functional redundancy may be widespread. In this study, the 10^{0} treatment was inoculated with approximately 10^{8} cells, while the 10^{-6} treatment was inoculated with only 100 cells (AODC); yet, for all of the substrates measured, activity of the regrown communities was the same. This means that the ~ 100 cells used to inoculate the 10^{-6} flasks must have contained a suite of genes sufficient to produce metabolic enzymes similar to the more diverse community, with regards to the substrates tested.

Another possible interpretation of these results is that the similarity in function across treatments was not the result of functional redundancy, but simply reflected a similarity in community composition; it is possible that the subset of organisms common

to all treatments/dilutions happened to be responsible for the particular functions examined. However, we feel that this is not likely - given the variety of substrates used, the wide range of treatments/dilutions studied, and the results of the various structural assays (AFLP, T-RFLP, and CLPP). Each of these structural assays showed a large difference in community structure among treatments, particularly when comparing the two ends of the dilution series (10^0 and 10^{-6}). For example, using AFLP, the average relative similarity calculated among communities within these treatments was quite high (10^0: 0.50, 10^{-6}: 0.63) compared to the relative similarity between treatments (10^0 to 10^{-6}: 0.13).

The results of our study are similar to the findings of Salonius (1981), who used dilution to reduce diversity of a soil microbial community. After inoculating the dilutions into sterile soil, he monitored net respiration (O_2 uptake) for five months. In general, respiration was the same for each of the regrown communities, except when richness was decreased below a critical level; in treatments with a very low diversity (just above the extinction point), respiration was severely impaired. Specifically, O_2 uptake was similar for communities regrown from undiluted, 1/5, and 1/10 dilutions of a forest soil community, but was diminished in the 1/100 dilution; no microbial growth occurred from the 1/1000 dilution treatment. Griffiths et al. (1997) reported similar results for a set of batch culture experiments. In that case, changing the evenness of a community had no impact on function, which was assessed by comparing the spectra of volatile organic compounds produced by the microorganisms. Several additional studies have been performed that used chloroform fumigation for increasing periods of time as a way to

progressively destroy species and manipulate microbial diversity in soil microbial communities (Degens, 1998; Dickens and Anderson, 1999; Griffiths et al., 1997 and 2000; Zelles et al., 1997). That work also demonstrated that there can be changes in microbial community structure with no change in function (Degens, 1998), but that function can be affected below certain levels of species richness (Griffiths et al., 1997; Griffiths et al., 2000). A similar phenomenon might have been observed in the present study if even more dilute treatments had been maintained (e.g., 10^{-7} and 10^{-8}, which would have been inoculated with 10 cells and 1 cell respectively).

The two DNA fingerprinting techniques (T-RFLP and AFLP) used to characterize microbial community structure in this study produced slightly different results, and seemed to differ in their resolution and sensitivity to different aspects of community structure (e.g., richness and evenness). T-RFLP was used to analyze the microbial communities based on variability in the 16S ribosomal RNA gene, and showed a major difference in community structure between the very dilute (10^{-5} and 10^{-6}) treatments (Figure 4.3 B) and all of the others. The T-RFLP profiles for the 10^{-5} and 10^{-6} communities contained several unique T-RFs, which were responsible for the separation of these treatments from the others in the PCA. The organisms corresponding to these T-RFs were likely present in all of the treatments, but were too rare in the less dilute/more diverse communities to be detected with the T-RFLP procedure; it is well known that populations that are not numerically dominant are not represented in the T-RFLP profiles if their template DNA is too small a fraction of the total community DNA pool (Dunbar et al., 2000; Liu et al., 1997). The detection of the unique T-RFs in the high-dilution

treatments suggests that the evenness of the community increased in the communities regrown from the more dilute inocula. This is consistent with numerical simulations that predict an increase in community evenness for inoculum created by high-dilutions (Franklin et al., 2001).

AFLP is a technique in which a restriction digest is performed on a DNA sample (similar to RFLP), and then a set of primer-recognition sequences (adaptors) is used to amplify the restriction fragments using PCR (Zabeau and Vos, 1993). In contrast to T-RFLP, the primers and restriction enzymes used are not specific for a given gene or group of genes but can, theoretically, interact in numerous random places throughout a genome. This makes AFLP a particularly useful technique for analysis of *overall* differences between communities, including strain- or species-level changes. Because T-RFLP is insensitive to changes in community composition that may occur at the level of individual strains or species (Buckley and Schmidt, 2001), microbial communities whose overall structure appears similar by T-RFLP analysis may still possess ecologically significant differences in community composition.

The AFLP results presented here show a strong difference in overall community structure between the communities at either end of the dilution series (either low dilution: 10^0 with 10^{-1}, or high dilution: 10^{-6}). Overall, the results from T-RFLP and AFLP were similar, except that the separation of the low-dilution communities (10^0 with 10^{-1}) was not reflected in the T-RFLP profiles. The separation of these low-dilution communities in the AFLP analysis was likely related to the large decrease in richness of the inoculum that occurred as the a result of the first few dilutions, and is consistent with previous

analysis of community structure in dilution/regrowth experiments (Franklin et al., 2001). However, probability suggests that the identity of the dominant organisms should not change much in these first few dilutions; since T-RFLP primarily detects dominant organisms, it was not able to distinguish any differences in community structure for the lower dilution/higher diversity treatments. The different, but complementary, results from the AFLP and T-RFLP analyses highlight the importance of using multiple techniques to evaluate microbial communities in ecological studies. This approach allowed us to detect differences in community structure that matched well our expectations based on hypothesized changes in both community richness and evenness.

In order to determine whether there was a relationship between community function and the observed changes in microbial community structure, a correlation analysis was performed between each functional parameter (uptake and efficiency for each substrate) and each PC from the genetic analyses. For AFLP, the first four principal components were examined (total variance explained: 51 %), and several significant correlations ($p < 0.05$) were found (Table 4.1). All of the significant correlations were associated with either glucose or acetate metabolism, and the different PCs were not linked with different levels of amino acid, citrate, or palmitic acid function. Several additional nearly significant correlations ($0.05 < p < 0.10$) were also found between glucose and acetate and PC 2, PC 3, and PC 4 (Table 4.1). However, no significant correlations were observed between the functional parameters and any of the first four PCs from the T-RFLP (total variance explained: 63 %; results not presented, $p \geq 0.10$).

Table 4.1. Pearson's correlation coefficients between each functional parameter and each PC derived from the AFLP analysis of community structure. The percent of variance explained by each PC is included.

Functional parameters	AFLP community structure analysis			
	PC 1 (17 %)	PC 2 (12 %)	PC 3 (12 %)	PC 4 (10 %)
Amino acid uptake	0.32	- 0.01	0.32	0.41
Amino acid efficiency	0.03	0.29	0.15	0.15
Citrate uptake	0.41	0.01	0.24	0.27
Citrate efficiency	0.14	- 0.26	0.33	0.40
Acetate uptake	0.26	- 0.63**	0.43	0.32
Acetate efficiency	0.47*	- 0.22	0.15	0.58**
Glucose uptake	0.47*	- 0.46*	0.60**	0.11
Glucose efficiency	0.06	- 0.51*	0.54*	0.17
Palmitic acid respiration	- 0.14	0.03	0.13	0.22

* Significant at $\alpha = 0.10$
** Significant at $\alpha = 0.05$

Overall, these results indicate that glucose and acetate metabolism may be more sensitive to variations in community structure and diversity than the other substrates we considered. This is somewhat unexpected, given that glucose and acetate are generally regarded as "universal" compounds (King, 2002), and are both central elements of polysaccharide metabolism and transformations of organic matter. Even though there were no significant differences between treatments with regards to acetate or glucose uptake (Figure 4.4 B and 4 D), there is a trend in the data that suggests these functions may have been suppressed in the more dilute/less diverse treatments. This trend is not visible for any of the other function assays, and none of the other substrates showed a significant correlation with community structure.

The PCA condensed the complex DNA fingerprints into a small number of principal components, and each of these derived variables describes a different aspect of the microbial communities' overall genetic composition. Each PC correlates with a distinct pattern of variability, which manifests as different groups of AFLP bands or T-RFs, and these different sets of bands/fragments are likely related to the distribution and relative abundance of different populations. The results of this correlation analysis suggest that changes in specific populations or groups of organisms in the microbial communities may be associated with changes in community function for certain substrates. For example, a strong positive correlation was detected between AFLP PC 3 and glucose uptake ($r = 0.60$, $p = 0.02$); higher factor scores on PC 3 were associated with higher community uptake rates for glucose. Unfortunately, since it is not possible to

relate the size of an AFLP fragment/band to any species or genus identification, these results do not provide any direct insight into the identity of the specific organisms responsible for the observed differences in community structure and function. In contrast, each T-RF in the T-RFLP theoretically corresponds to a particular type/species of bacteria, and it may be possible to identify the particular community members associated with each T-RFLP PC using clone libraries and DNA sequencing. However, given that the T-RFLP results presented here did not show any significant correlations with community function, we did not make use of this approach.

The fact that there were no significant correlations between T-RFLP community structure and community function, but strong correlations for AFLP, suggest that the analysis of overall community structure using AFLP may be a better predictor of potential/actual changes in community function, compared to an analysis of the 16S rRNA gene (via T-RFLP). Given that metabolic functions are seldom phylogenetically grouped (Ward et al., 1995), this is not necessarily surprising. Because AFLP fingerprints reflect variability present throughout the entire community DNA pool, differences in functionally relevant genes can also contribute to the AFLP profile, and thus may explain the correlation observed between the AFLP community structure assay and glucose and acetate metabolism. Another major difference between AFLP and T-RFLP is the fact that rarer organism types are typically not detected by T-RFLP (discussed above), and an alternate explanation for the results presented here is that the correlation between AFLP-based community structure measurements and activity is partially due to the inclusion of these organisms in the structural assay. This is

particularly important considering the fact that so many of the methods currently used to assay microbial communities focus solely on the characterization of the dominant organism types. Those organisms may represent a small portion of the total microbial community; for example, in water only about 10 % of the simultaneously coexisting species are dominant (Torsvik et al., 2002). The development of techniques that better consider the contribution of rare organism types to overall community structure and function is an important area for further study.

One of the limitations of this study is that community function was only determined for a small group of substrates. However, the compounds were chosen to represent a range of different types of chemical groups: amino acids, carboxylic acids (short (acetate and citrate) and long chained (palmitic acid)), and carbohydrates (glucose). It is certainly possible that differences in function might have been found if the communities had been presented with more exotic compounds. In fact, we originally selected the palmitic acid ($CH_3(CH_2)_{14}COOH$) and benzene so that such a comparison could be made. Unfortunately, no data were obtained from benzene, as discussed in the Methods section. The results for palmitic acid show that mineralization was high, relative to the mineralization rates of the other substrates, and it is important to point out that this means the similarity in function across treatments for this substrate was not the due to an inability of the community to metabolize the compound. In a similar study, Yin et al. (2000) analyzed the functional redundancy of a set of soil communities, using four specific substrates (L-serine, L-threonin, sodium citrate, and α-lactose hydrate), and found that functional redundancy did not necessarily correlate with total community /

richness and diversity. Moreover, they found no difference in redundancy across the different substrates; the number and diversity of bacterial groups able to respond to each substrate at each location was similar.

There is considerable debate among ecologists as to what processes should to be chosen to best characterize ecosystem or community functioning (Ghilarov, 1997; Gitay et al., 1996), and the study reported here only addresses the metabolic uptake of a small group of compounds. Ideally, a more complete analysis of the function of microbial communities would include measures of other compounds and processes, and would also evaluate community stability (resistance and resilience). There is some evidence that suggests that well-defined microbial functions such as nitrification and methane oxidation, which are carried out by a limited microbial sub-set, may be more sensitive to changes in diversity than broader scale functions such as respiration or decomposition (Griffiths et al., 2000; Toyota et al., 1999; Wu et al., 2002). Kandeler et al. (1996) also showed that carbon cycling may be less sensitive to changes in microbial community composition than nitrogen and phosphorus dynamics.

Though dilution is typically thought of as a linear process (which is appropriate for some applications), the response of diversity to dilution in non-linear, and numerical simulations have demonstrated that the impact of dilution can vary depending on the diversity (richness and evenness) of the original community (Franklin et al., 2001). For example, if the original community has an even distribution, or contains very few rare types, the dilution procedure will have little impact on the composition of the inocula. Each successive dilution step will simply decrease the number of organisms, but will not

change the relative abundance or identity of the community members. At very high dilutions, when the number of cells being transferred is less than the richness of the original community, diversity will decrease significantly as organism types are excluded from the mixture. In situations where the original community is highly dominant, inoculum richness will decrease greatly with the first few dilution steps (probability suggests that few of the rare organisms would be transferred), and evenness will increase. Smaller changes in diversity are expected at intermediate dilutions until, as discussed above, the number of cells being transferred in the dilution is less than the number of organism types.

The actual experimental communities regrown from the diluted mixtures were not expected to exactly mimic these simulations. The dilution procedure may have disrupted some of the biological interactions among organisms (e.g., synergistic or mutualistic relationships, competition), which could lead to a change in community structure with regrowth. Likewise, the diversity of the regrown community may have been influenced by variability in growth rates among organism types. Nonetheless, some conclusions about the diversity of the original sewage microbial community used in this study can be made by examining the results of the dilution/regrowth procedure in the context of these numerical simulations. In particular, the fact that there was a large change in community structure after the initial dilution indicates that the original community did contain a number of rare organism types. Moreover, community structure was expected to change at high dilutions when the number of cells being transferred in the dilution is less than the number of organism types; in this study, a shift in community structure and composition

was generally observed between the 10^{-5} and 10^{-6} dilution treatments, which suggests that there were between 100 and 1000 types of organisms in the original community. This is consistent with other estimates of microbial diversity in sewage samples, which range from 70 types per ml (Curtis et al., 2002) to approximately 1000 types per ml (Franklin et al., 2001), but much lower than has been found in pristine soil, which may contain tens of thousands of different types (Curtis et al., 2002; Torsvik et al., 2002).

Despite growing knowledge of the magnitude of prokaryotic diversity, most of the organisms in natural environments are uncultivated, and their functional roles and abilities remain unknown. The fact that most questions about the structure and function of microbial communities require relative comparisons, which can be made at the community level, helps to overcome this problem, particularly if multiple analytical techniques are employed to increase the robustness of the relative comparisons (Hughes et al., 2001). Most of the interest in studying the relationship between microbial diversity and function is based in the assumption that diversity may influence ecosystem stability and productivity, and could help mediate a community's response to stress and perturbation. In the study presented here, we examined the relationship between microbial diversity and function for a sewage microbial community and found that functional redundancy may play an important role in the stability of microbial communities, even for communities with a relatively low diversity such as sewage. The dilution/regrowth approach we employed was successful in creating communities that differed in both overall structure and diversity, but no significant changes in the *in situ* function of the communities were observed for any of the substrates we considered. The

data did suggest that the activity of the microbial community with regards to glucose and acetate metabolism may have been associated with specific changes in community structure, and a significant loss of function might have been observed if additional treatments, with lower levels of diversity, had been maintained. Further research into the relationship between microbial community structure and function may help microbial ecologists to determine what role, if any, microbial diversity may play in the environment and to determine what impact functional redundancy may have on ecosystem function and stability.

Chapter 5.

The distribution of microbial communities in anaerobic and aerobic zones of a shallow coastal plain aquifer.

Franklin, R. B., D. R. Taylor, and A. L. Mills. 1999.
Microbial Ecology. 38:377-386.

Abstract

Randomly amplified polymorphic DNA (RAPD) fingerprinting was used to determine the genetic similarity of whole-community DNA extracts from unattached microorganisms in several groundwater wells. The study site was a shallow coastal plain aquifer on the Eastern Shore of Virginia that contains distinct regions of anaerobic and aerobic groundwater. Several wells in each region were sampled, and principal component and cluster analyses showed a clear separation of the microbial communities from the two chemical zones of the aquifer. Within these zones, there was no relationship between the genetic relatedness of a pair of communities and their spatial separation. Two additional sets of samples were taken at later times, and the same clear separation between communities in the different zones of the aquifer was observed. The specific relationships between wells within each zone changed over time, however, and the magnitude and direction of these changes corresponded to concurrent changes in the groundwater chemistry at each well. Together, these results suggest that local variation in groundwater chemistry can support genetically distinct microbial communities, and that the composition of the microbial communities can follow seasonal fluctuations in groundwater chemistry.

5.1. Introduction

The microbiology of aquifers and subsurface sediments is a subject of expanding interest, in part because aquifers are a major source of freshwater in many countries. These undergroundwaters represent 97% of all global freshwater and, though they are commonly exploited for drinking water, agriculture, and industry, they are poorly understood ecosystems (Gilbert et al., 1994). Studies of shallow subsurface waters have found substantial numbers of microorganisms, predominantly prokaryotic, and have shown that these communities may perform a number of significant functions that may dramatically affect the chemical composition of the groundwater (see review in Gounot (1994)). Much of this research has been stimulated by concern over contamination of groundwater supplies, particularly organic contamination from industrial operations, and the potential for microbial degradation of such pollutants as a means of restoring and purifying these waters (Atlas, 1981; Atlas and Bartha, 1992; Madsen and Ghiorse, 1993; Semprini et al., 1990).

Numerous studies have detailed the groundwater habitat, focusing on the abundance (see references in Ghiorse and Wilson (1988) and Madsen and Ghiorse (1993)), morphology (Balkwill, 1989; Bone and Balkwill, 1988), physiological state (Beloin et al., 1988; Bengtsson, 1989; White et al., 1983), and genomic diversity (Jiménez et al., 1990; Stim et al., 1990) of its microbial residents. Research emphasis has recently shifted away from isolate and culture-based approaches, toward community-level analyses, where entire microbial communities are used as the functional units of study. Such investigations have considered rates of metabolism of specific substrates by

entire communities (Beloin et al., 1988; Federle et al., 1990; Long et al., 1995), performance of specific activities (e.g., denitrification (Smith and Duff, 1988)), and expression of certain genes (Wilson et al., 1999). Overall community structure in groundwater has also been compared using community-level physiological profiling (CLPP) (Colwell and Lehman, 1997; Lehman et al., 1995), phospholipid fatty acid profiling (Kieft et al., 1997), and 16S ribosomal RNA gene sequencing (Pedersen et al., 1996).

Microbial community structure in groundwater systems may be influenced by a number of factors including site history, biological interactions (e.g., synergistic/mutualistic relationships (Chapelle, 1993), competition (Chapelle and Lovley, 1992), and predation (Sinclair and Ghiorse, 1987)), and physical habitat variation. The distribution of community members has also been correlated with changes in the groundwater chemical environment (e.g., availability of organic and inorganic carbon, dissolved oxygen, sulfur, nitrogen, phosphorus, and iron (Balkwill, 1989; Bengtsson, 1989; Chapelle, 1993; Marxsen, 1988)). However, when such studies evaluate how these different types of variables influence microbial community structure, they usually do so at a very broad scale, and rarely consider how the spatial separation of organisms within a system may influence population interactions and community dynamics. There likely exists a coupling of distance with community relatedness (a microbial community patch size) at spatial scales below that of the aforementioned physical, chemical, and biological variables. Studies in other environments suggest that the scale of bacterial patchiness can

be quite small; for example, the patch size of a microbial community in agricultural soils was estimated to be approximately one meter (Balser and Firestone, 1996), and marine bacterioplankton communities exhibit strong patchiness at the centimeter scale (Duarte and Vaqué, 1992).

The present study examined two chemically distinct zones of a shallow aquifer (well-defined regions of low-oxygen and aerobic groundwater) to evaluate the extent to which differences in aquifer redox chemistry may influence the genetic structure of the resident microbial communities. At the research site, the proximity of the two zones of groundwater flow, and the fact that they percolate through sediment from a similar depositional environment, eliminated the need to consider differences in macro-environment (e.g., rainfall and climate or variation in sediment properties) that may also influence microbial community development in the subsurface. Within these two zones, a fundamental question motivating the research was whether the scale of microbial community relatedness occurred on a scale similar to that of the major chemical differences (i.e., aerobic versus anaerobic conditions).

Several samples were collected within each chemical zone of the aquifer and randomly amplified polymorphic DNA (RAPD) was used to compare genetic community structure and estimate percent similarity among the different communities (Franklin et al., 1999a). RAPD analysis showed that the communities in the anaerobic and aerobic regions of the field were quite different. Some temporal variation in community structure was observed and those changes paralleled fluctuations in the groundwater chemistry of

the wells over the same time period. There was little correlation between community relatedness (percent similarity between wells) and spatial separation of the sampling locations, either throughout the entire field, or within each region. Although the communities were distinctly different in the two regions of the field, community patch size was smaller than the smallest sampling separation distance, viz. 10 m.

5.2. Materials and methods

5.2.1. Site descriptions and sampling schedule

The research site is a shallow coastal plain aquifer on the lagoonal shoreline of Virginia's Eastern Shore, located in a small (1.7 ha), abandoned agricultural field. This surfacial aquifer is approximately 24 – 30 m thick, and the depth to the water table across the sloping field varies from 1 m to about 6 m. A distinct zone of oxygen-depleted groundwater surrounds a mass of buried vegetable waste, from a tomato cannery, on the far north side of the field (Figure 5.1). The anoxic conditions (below 0.5 mg liter^{-1} dissolved oxygen) extend down-gradient, while the water in the rest of the field is aerobic, consistent with the regional groundwater. Dissolved oxygen concentration in the aerobic region varies seasonally between 5 and 11 mg liter^{-1} (Knapp, 1997). In the zone we have termed anaerobic, dissolved oxygen concentrations are always at or near 0 (always below 1 mg liter^{-1}), except for occasions when large storms bring oxygenated water downward to the surface of the aquifer. Other chemical analyses have shown that these two regions of the field differ in the amount of nitrate, alkalinity, ammonia, and dissolved iron present (Table 5.1) in a manner consistent with oxygen depletion and

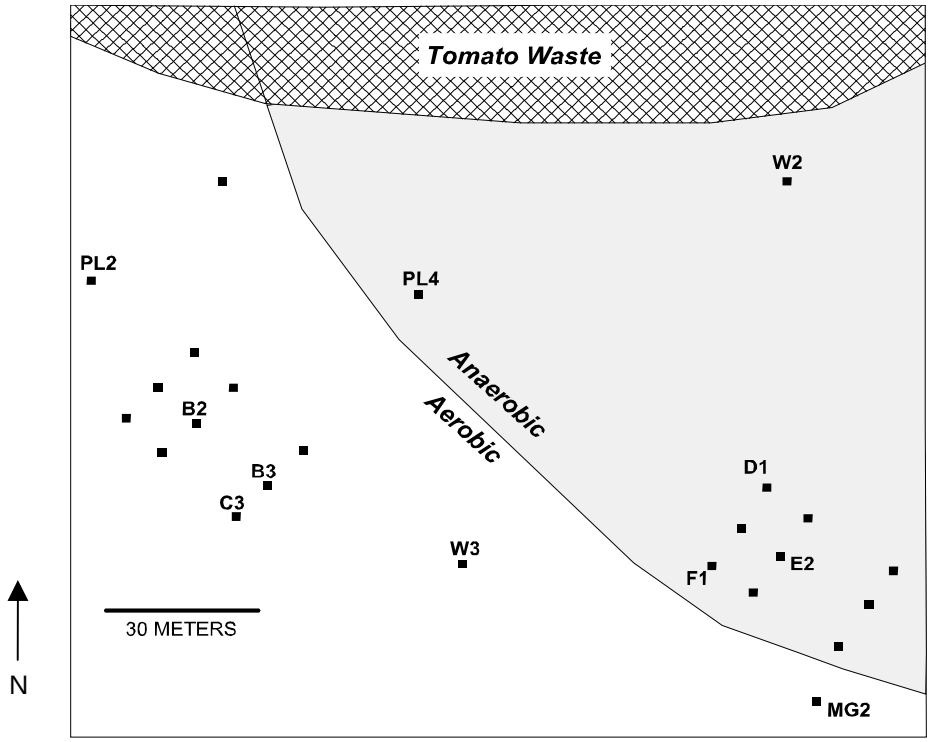

Figure 5.1. Map of the Oyster field site displaying sampling wells and regions of aerobic and anaerobic groundwater. The hydrologic gradient at the site points roughly eastward. To the north lies the organic contamination, buried vegetable waste from a tomato cannery.

Table 5.1. Concentrations for a number of groundwater constituents in the aerobic and anaerobic portions of the aquifer. The range presented represents observed values from 16 sampling efforts between June 1994 and November 1996. Data from Knapp (1997).

Parameter	**Aerobic Zone[1]**	**Anaerobic Zone[2]**
pH	5.3 - 6.5	5.9 - 6.6
Alkalinity (mg liter^{-1} HCO_3^-)	18.3 - 39.7	150 - 384
DOC (mg liter^{-1})	1.72 - 4.47	2.22 - 5.86
Dissolved Oxygen (mg liter^{1})	5.0 - 10.6	< 0.9
Total Dissolved Iron (mg liter^{-1})[3]	0.0001 - 0.01	12 - 42
Sulfate (mg liter^{-1})	28.8 - 36.5	26.2 - 44.2
Nitrate (mg liter^{-1})	40 - 50	0 - 1
NH_4^+ (mg liter^{-1} N)	0	0.434 - 2.42

[1] These values for groundwater chemistry are from well PL2, an aerobic well ~55 m directly up-gradient from wells C3 and B3 that were sampled in this project.

[2] Values from well D1.

[3] Samples were filtered and acidified in the field, returned to the laboratory and passed through a cadmium column to reduce Fe^{3+} to Fe^{2+} followed by colorimetric analysis by the Ferrozine assay (Stookey, 1970).

reducing conditions. Moreover, average microbial abundance, measured by acridine orange direct counts (AODC), differs between the two zones and higher concentrations of cells are found in the anaerobic zone (well D1: 5×10^6 cells ml^{-1}; well W2: 3×10^7; wells C3 and B3: 3×10^5 (Lancaster and Mills, 1995)).

Several groundwater wells, constructed of 2-inch diameter PVC, have been installed throughout the field (Figure 5.1), and nine of them were chosen for use in this study. Wells B3, C3, W3, and MG2 (in the aerobic zone), and wells F1, E2, D1, W2, and PL4 (in the anaerobic zone) were sampled in August 1997. Four of these wells, two aerobic (B3 and C3) and two anaerobic (D1 and W2), were further sampled in June 1997 and January 1998.

5.2.2. Sample collection

To isolate the microbial community for analysis, water samples were concentrated by filtration onto 0.22-μm pore-size polycarbonate membranes after pre-filtration through AE glass-fiber filters. Prior to sample collection, each well was purged for 10 - 15 min. For the June 1997 sampling, approximately 20 liters of water were filtered, onto a single filter. However, preliminary analysis of these samples indicated that the amount of DNA obtained was far in excess of that needed for RAPD community profiling, so further sampling efforts focused on replication rather than collecting large volumes of water. In August 1997 and January 1998, three replicate samples of approximately 5 liters each were filtered at each well.

After each sampling, filters were quickly frozen in dry ice and ethanol (within 5 hours of collection), transported to the lab on dry ice, and stored at -80°C. Filters were later processed and whole-community DNA was extracted as described elsewhere (Franklin et al., 1999a). An additional purification step, using the High-Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN), was added to the original procedure and the isolated DNA was resuspended in 100 µl of 10 mM Tris buffer (pH 8.5).

5.2.3. RAPD

After extraction of whole-community DNA, RAPD amplification reactions were carried out in a volume of 25 µl using the procedure suggested by Williams et al. (1990) with slight modification. A 5 µl portion of a DNA solution was added to a 20 µl reaction mixture containing: 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM primer, and 1 unit of *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT). Reaction mixtures were covered with one drop of mineral oil and amplifications were performed in a Hybaid PCR Express Thermal Cycler programmed for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. PCR products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed under UV light.

The amount of DNA entering each PCR reaction was standardized so that differences in the concentration of cells at each groundwater well would not artificially bias the RAPD results. For the June samples, the concentration of DNA was estimated using PicoGreen dsDNA quantification reagent (Molecular Probes, Eugene, OR) and approximately 400 pg of DNA were used in each PCR reaction. DNA yields from August and January were expected to be much lower, based on the smaller volume of water sampled, and, because of the desire not to sacrifice sample for quantification purposes, standard spectrophometric/spectrofluorometric quantification procedures were not used. Instead, the DNA concentration was normalized across all August and January samples based upon the number of bacterial cells entering the extraction procedure (Franklin et al., 1999a). Extractions were performed on approximately 10^8 cells.

Several arbitrary primers, purchased from Operon Technologies (Alameda, CA), were used to profile all three sets of samples: C4 (5' CCGCATCTAC 3'), D5 (5' TGAGCGGACA 3'), F4 (5' GGTGATCAGG 3'), F1 (5' ACGGATCCTG 3'), F14 (5' TGCTGCAGGT 3'), S10 (5' ACCGTTCCAG 3'), and T7 (5' GGCAGGCTGT 3'). Additionally, primer F3 (5' CCTGATCACC 3') was used for the June and August samples, and primers F7 (5' CCGATATCCC 3') and S14 (5' AAAGGGGTCC 3') were also used for the June samples. This resulted in an overall comparison of 70 bands in June, 97 bands in August, and 76 bands in January. Within a set of samples, nearly all of the bands were variable (8 % were present in all samples screened), and an individual well sample contained between 18 and 42 bands.

5.2.4. Data analysis

For each primer, each band observed on the agarose gel was treated as a unit character and scored as present or absent in each sample. The data sets from each primer were then consolidated and a distance matrix was calculated using the Jaccard coefficient (Sneath and Sokal, 1973). Dendrograms were constructed using UPGMA clustering and a bootstrapping procedure was used to assess the significance of the groupings and subgroupings in each dendrogram (Stackebrandt and Rainey, 1995; Swofford et al., 1996).

In this research, the bootstrapping was accomplished by first using "SEQBOOT" in PHYLIP (Version 3.5 c) to bootstrap the presence/absence (1/0) data sets 100 times (Felsenstein, 1993). Each of the resultant data sets was then fed into the clustering program of SPSS (Version 8) and similarity matrixes were determined using Jaccard's coefficient. Next, distance matrices (Dissimilary = 1 – Similarity) were computed and the "NEIGHBOR" subroutine of PHYLIP was used generate 100 different recomputed trees. The "bootstrap value", the proportion of recomputed trees that contain a given node, was then determined by feeding the tree file from "NEIGHBOR" into the "CONSENSE" subroutine of PHYLIP.

Principal component analyses (SPSS, Version 8) of the original data were also performed and, diagrams of the first two principal components were constructed. Though PCA is not typically recommended for use with binary data such as these, it is often used as an alternate means of *visualizing* the relationships from the different RAPD profiles (Demeke and Adams, 1994; Franklin et al., 1999a; Wikström et al., 1999).

To compare the spatial distribution of the microbial communities in the field, plots were made of relative community similarity (as determined by the Jaccard coefficient) versus distance between sampling locations. Additional graphs were made to evaluate this relationship separately in the aerobic and anaerobic zones. An analysis of covariance (ANCOVA) was then used to determine whether the relationship between a pair of communities was more influenced by chemical zone (are the two samples from the same or different chemical regions of the field?) or by spatial separation. Average genetic similarity between each pair of communities was used as the dependent measure and the distance between each pair of wells was the covariate. Chemical "zone" was coded into three groups: 1 - both communities sampled from aerobic wells, 2 - both communities sampled from anaerobic wells, and 3 - samples compared from different chemical zones of the field.

5.3. Results

5.3.1. Spatial distribution of communities within the field

5.3.1.1. Principal components and cluster analyses

Cluster analysis of all nine wells sampled in August showed a clear separation of the microbial communities in the anaerobic and aerobic zones of the aquifer; however, well E2 did not fall within either group (Figure 5.2). Although high bootstrap values were calculated for the internal nodes of the dendrogram, the larger groupings of "aerobic" and "anaerobic" were not well supported. This is because, in the different bootstrapping runs, well E2 moved between these two clusters, and the fluctuation of this

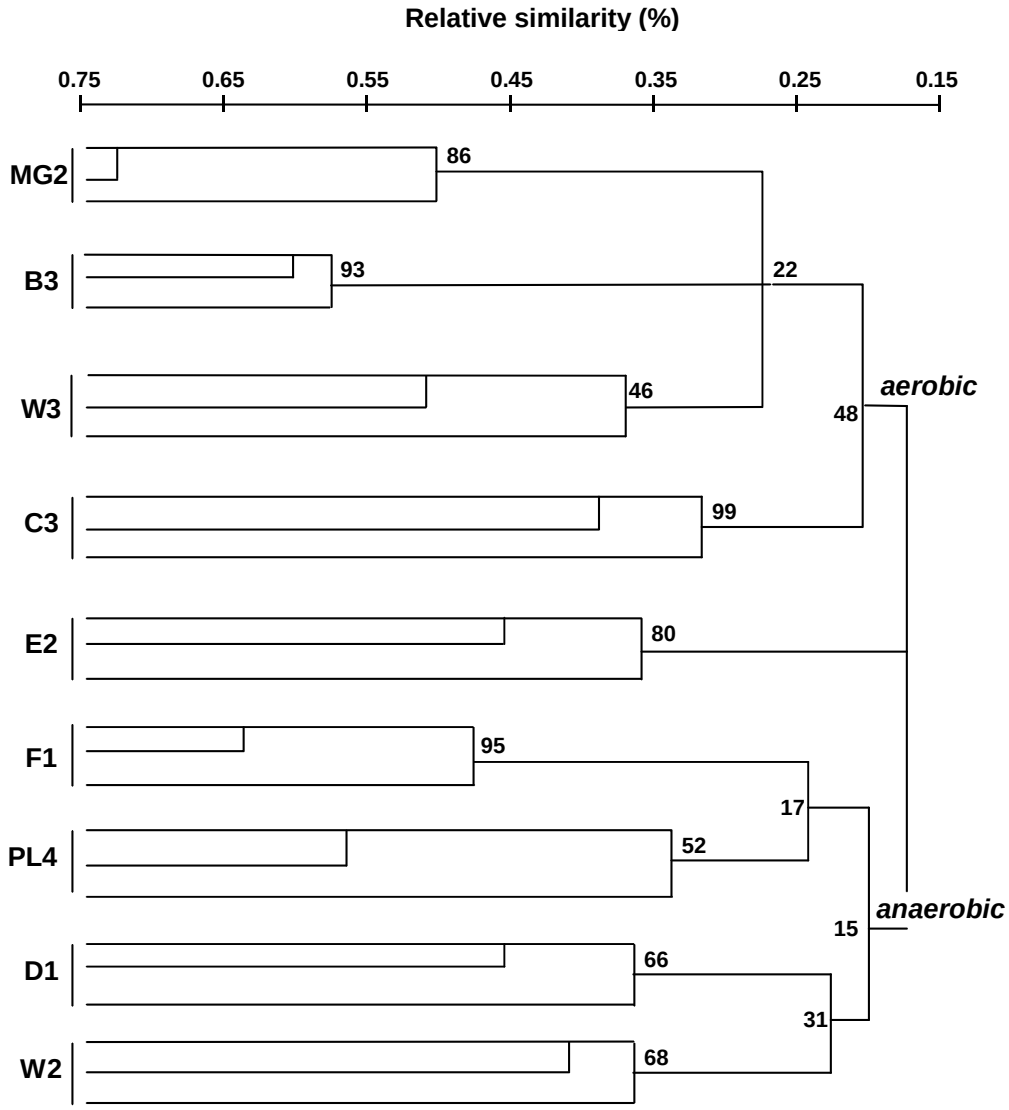

Figure 5.2. Dendrogram displaying the results of a cluster analysis of all nine wells sampled in August 1997. The scale along the top represents similarity, the three prongs for each well represent independent replicates (separate 5-liter fractions of water collected from each well), and the numbers at each node are bootstrap values (bootstrap performed using 100 replications).

single well accounted for the low bootstrap values associated with the two major clusters (aerobic/anaerobic (Figure 5.2)). When the E2 data were excluded from the cluster analysis, the same overall pattern of separation of the communities was seen but with higher bootstrap values (complete results not shown, see Figure 5.5B for example).

Considering well E2's groundwater chemistry, and the fact that it is physically positioned between wells F1 and D1, the expectation was that it would closely resemble the communities from the other anaerobic wells. Instead, the communities in E2 were equally similar to the communities from the aerobic or the anaerobic zones of the field. It is possible that a sampling error occurred at E2 and insufficient purging of the well prior to collection may have caused these puzzling results. If the well was not adequately purged, the microbes sampled may have been part of a different community, associated with the stagnant water in the well. Apart from this one well, the PC plot (Figure 5.3) shows a clear separation of the communities that correlates with the large change in dissolved oxygen availability. Communities from the aerobic wells have very low scores (negative) on PC 1, wells near the aerobic/anaerobic boundary have scores close to zero, and the wells in the anaerobic zone have positive values. When the PCA was rerun without the E2 data, tighter clusters formed and greater separation occurred between groups in the principal components plot.

In the cluster analysis, the communities from the aerobic wells were further divided into two subgroups (MG2, B3, and W3 separated from C3). However, this separation was poorly supported by the data (bootstrap value of 22) indicating that

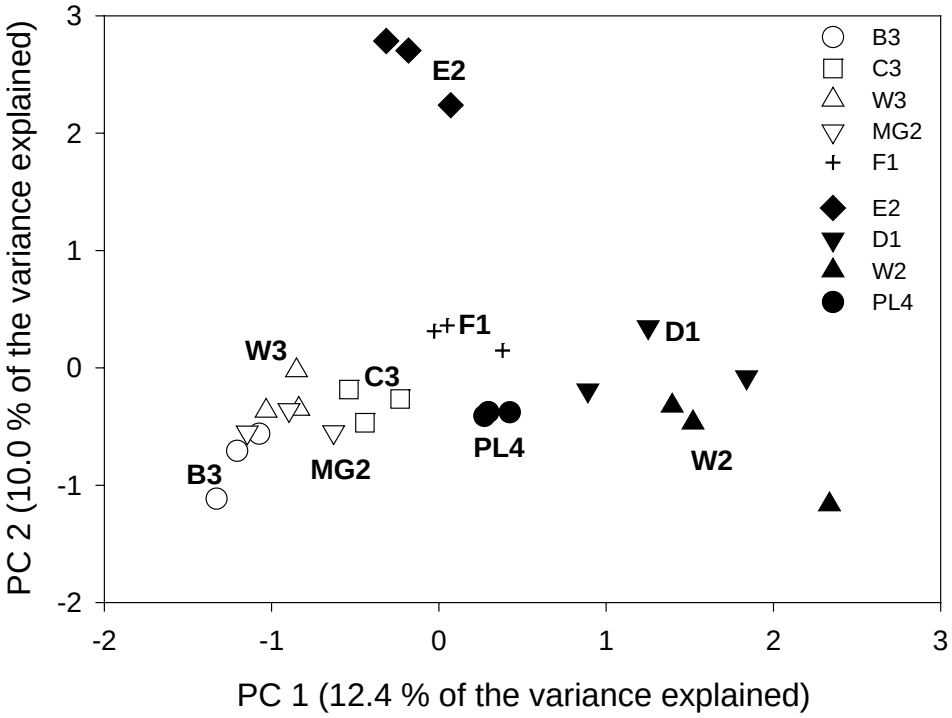

Figure 5.3. PCA of the RAPD profiles for all nine groundwater wells sampled in August 1997. The three points for each well represent independent replicates (separate 5-liter fractions of water collected from each well). The black symbols are samples collected from the anaerobic zone of the field; the white symbols represent aerobic wells. The percent of variance explained by each principal component is listed.

community structure did not differ greatly among the aerobic wells. This conclusion is further supported by the principal components analysis (Figure 5.3) that shows little separation of the communities from the different aerobic sampling locations.

Within the cluster of anaerobic wells (F1, PL4, D1, W2), the communities in wells F1 and PL4 grouped together as did D1 and W2 (Figure 5.2). Though neither of these subgroups was particularly well supported in the cluster analysis (bootstrap values of 17 and 31 respectively), they did separate on the first axis of the principal components plot, suggesting that these subdivisions may be relevant. Both F1 and PL4 lie near the boundary of the anaerobic/aerobic regions, where fluctuations in the water table could cause these wells to experience a wide range of environmental conditions. On the other hand, wells D1 and W2 lie deeper in the anaerobic zone, closer to the source of the organic contamination, and are most similar to one another.

5.3.1.2. Comparison of community relatedness and spatial separation

The spatial distribution of communities within the well field was compared by plotting average relative similarity between each community (as determined by the Jaccard coefficient) versus spatial separation (distance in meters). Because of the increased hydrological linkage and more similar water chemistry of spatial proximal wells, it was expected that genetic similarity would be high when the distance between communities was small, and would decrease when comparing more distant pairs of wells. In fact, no such relationship was found (Figure 5.4). Exclusion of the E2 data, as its comparison with other wells may result in a calculation of percent similarity that is

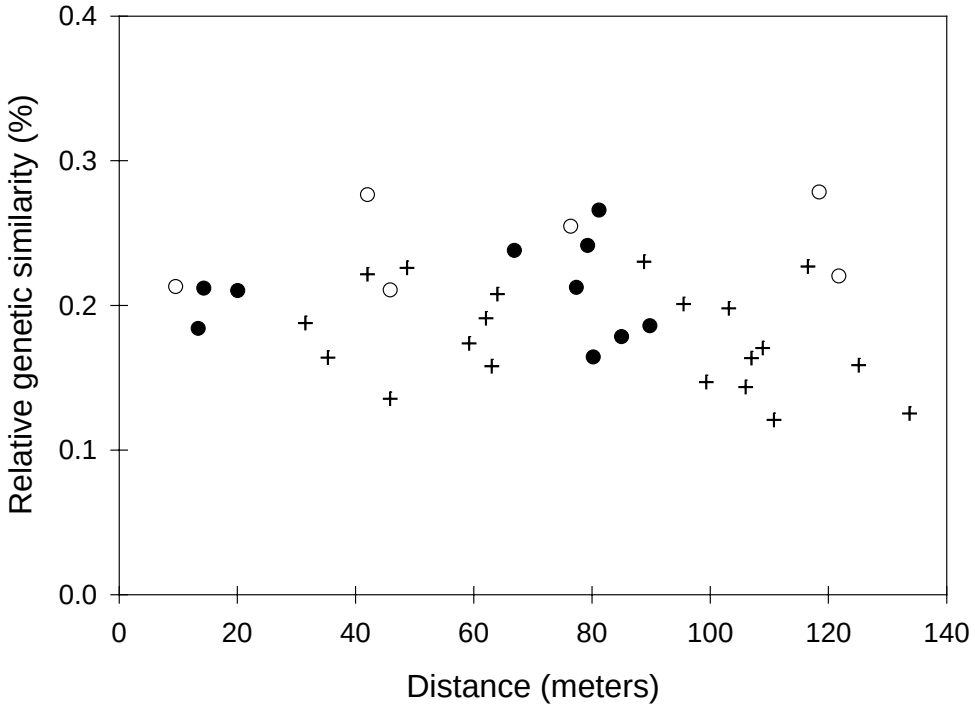

Figure 5.4. Plot of community genetic similarity verses distance between sampling locations for all samples from August 1997. The closed symbols represent distances and similarities calculated between aerobic wells, the open symbols represent values calculated between anaerobic wells, and the crosses represent values calculated between aerobic and anaerobic wells.

unusually low, had no effect. Furthermore, graphs made of the anaerobic and aerobic zones separately showed no relationship between community similarity and distance (results not shown).

To examine the relative importance of spatial separation versus groundwater chemistry on the observed patterns of community structure, an analysis of covariance (ANCOVA) was calculated comparing average similarity between communities. Each pair of communities was grouped as being from the same chemical zone, either both aerobic wells or both anaerobic wells, or as being from different zones. The average similarity for any two communities was greater if they were from the same chemical zone (average similarity for aerobic pairs: 0.27, anaerobic pairs: 0.21, different zone pairs: 0.18, $p = 0.0035$) but the influence of spatial separation on this relationship was not significant ($p = 0.621$). As with the cluster and PC analyses, these results suggest a more homogeneous distribution of communities in the aerobic zone of the aquifer.

5.3.2. Temporal consistency of anaerobic/aerobic patterns in community structure

Temporal variability of the microbial communities in these two zones of the aquifer was assessed by comparing four wells (two anaerobic (D1 and W2) and two aerobic (C3 and B3)) at three different times: June 1997, August 1997, and January 1998 (Figure 5.5). In all three cases, the communities from the aerobic and anaerobic zones separated, though the specific relationships among wells changed. In June and August, two distinct clusters formed, one aerobic (wells B3 and C3) and the other anaerobic (D1

and W2), and these were supported by high bootstrap values (Figure 5.5 A and B). In January, B3 and C3 formed a well-supported aerobic cluster (bootstrap value of 82) but the relationship of the anaerobic wells changed - W2 moved within the clade containing the aerobic wells, and D1 remained distinct (Figure 5.5 C).

To examine how the communities changed over time, the summer and winter samples were compared using a principal component analysis performed on the combined August and January datasets (Figure 5.6). Because a different concentration of DNA was used in the RAPD profiling of the June samples, a direct analysis comparing those results with the later samplings could not be made; however, August and January profiles were generated using the same starting concentration of DNA in the PCR reaction. Analysis of the pooled data showed that the profiles for the aerobic wells were consistent over time, but that the communities in both W2 and D1 changed between the two sampling dates. The communities in these two wells became more like the communities from the aerobic zone (W2 moved toward the aerobic communities on PC1, D1 moved through them on PC2 (Figure 5.6)).

Quarterly monitoring of several groundwater chemical parameters (including pH, alkalinity, dissolved oxygen, ammonium, phosphate, dissolved iron, calcium, magnesium, potassium, and manganese) has been taking place at this site for several years and Figure 5.7 shows a principal components plot comparing the Summer 1997 and Winter 1998 chemical samplings for wells D1, W2, W3, and B2 (L. Lancaster, unpublished data). Unfortunately, data were not collected for either B3 or C3, the two aerobic wells

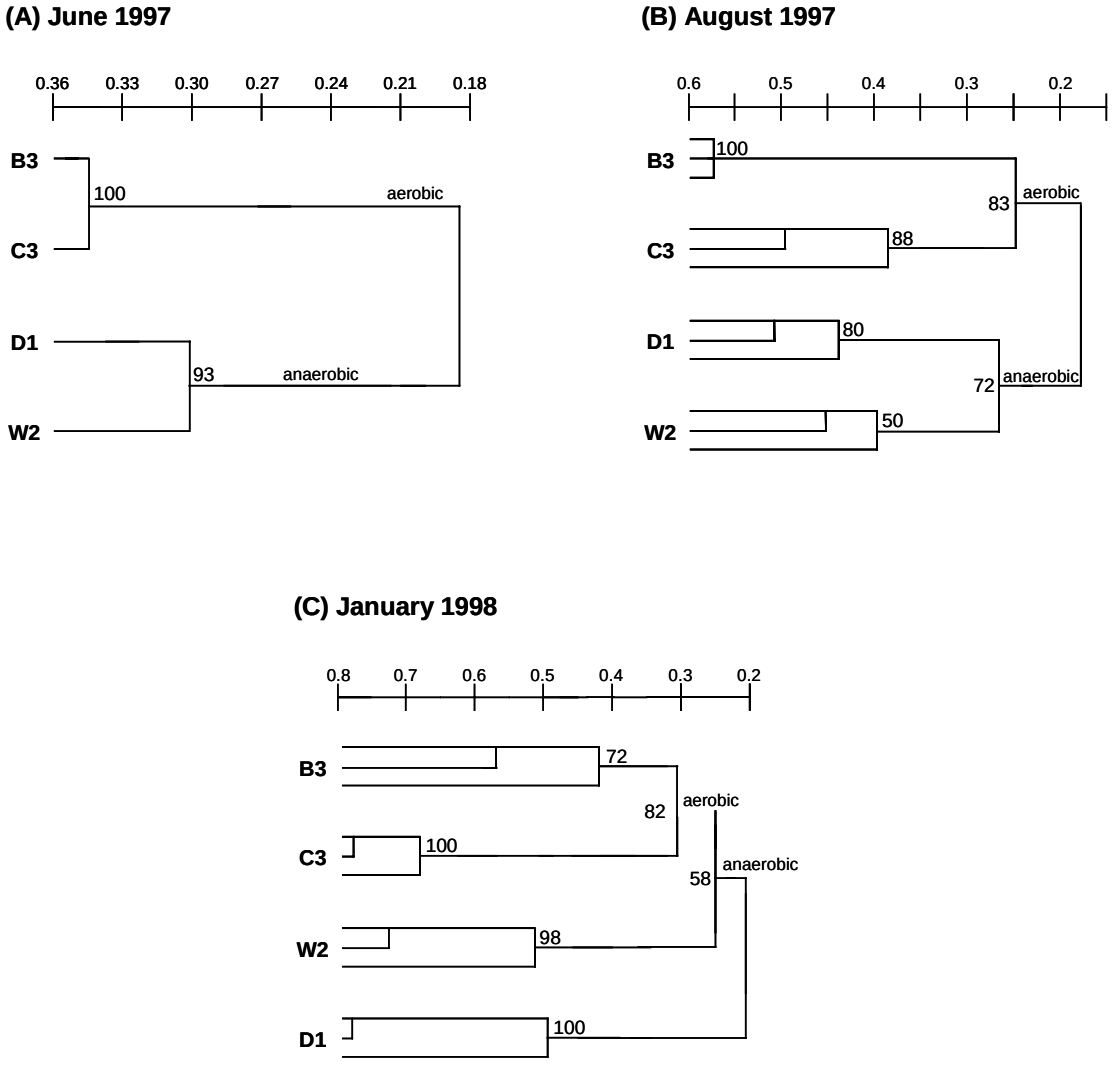

Figure 5.5. Dendrograms displaying the results of a cluster analysis of the four groundwater wells sampled for the temporal comparison. The scale along the top represents similarity and the numbers at each node are bootstrap values (bootstrap performed using 100 replications). (A) June 1997 (B) August 1997 and (A) January 1998. In (A) a single 20-liter sample was collected from each well. For (B) and (C) the three prongs for each well represent independent replicates (separate 5-liter fractions of water collected from each well).

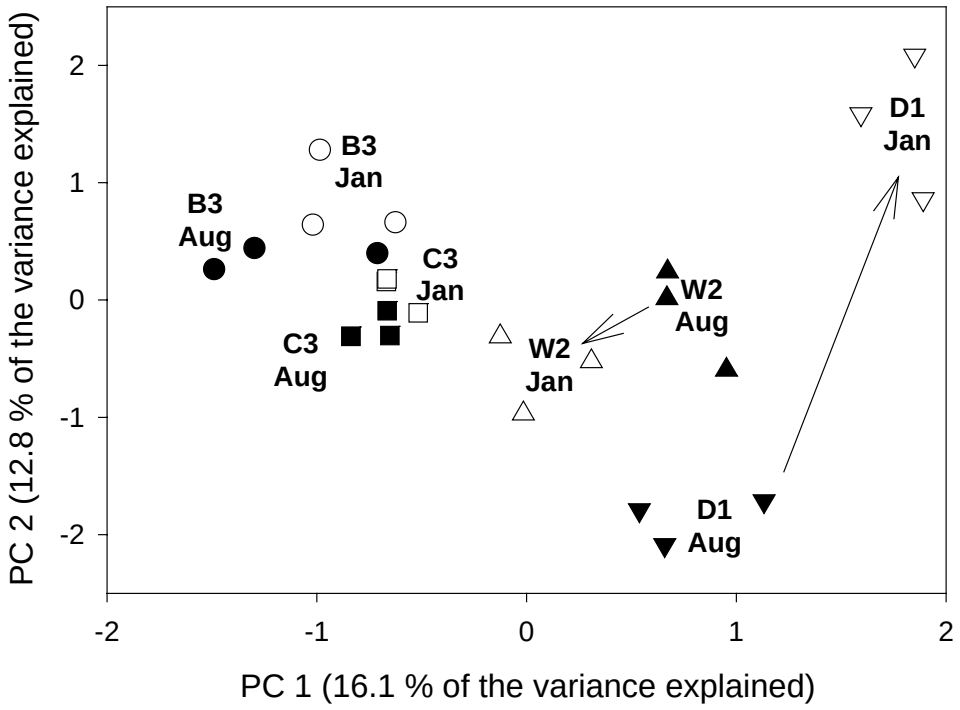

Figure 5.6. PCA of the RAPD profiles for the four groundwater wells compared in August 1997 and January 1998. The three points for each well represent independent replicates (separate 5-liter fractions of water collected from each well). The black symbols are from the August sampling; white symbols represent January. The percent of variance explained by each PC is listed.

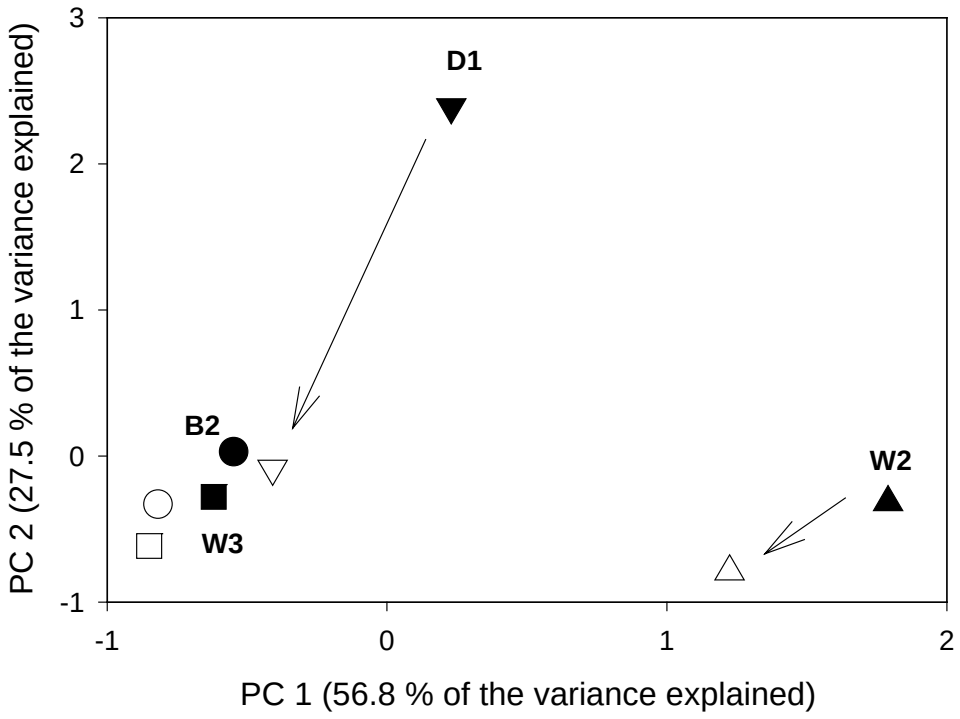

Figure 5.7. PCA of overall groundwater chemistry in Summer 1997 and Winter 1998. As in Figure 5.6, the filled symbols represent the Summer samples and the open symbols represent the following Winter's samples. The percent of variance explained by each PC is listed.

compared with RAPD profiling, so B2 and W3 were used as proxy measures of their chemical patterns (a reasonable substitution given the relative homogeneity of the water chemistry in the aerobic zone). The principal components plot shows that the chemical patterns in the anaerobic wells change over time, becoming more like what is seen in the aerobic wells - the same shift observed for the genetic composition of the community (Figure 5.6). The chemical parameters that loaded highly in the principal components analysis were: PC1 - pH, alkalinity, dissolved oxygen, iron, and soluble cations, PC2 - ammonium.

5.4. Discussion

In this aquifer, the distinct regions of aerobic and anaerobic groundwater supported different microbial communities. Within the aerobic zone, where the groundwater chemistry was fairly homogeneous, the communities from the various wells were quite similar. The degree of similarity among the wells in the anaerobic region was less than in the aerobic zone, and reflected the higher level of dissimilarity in the chemical conditions found in the anaerobic area. Beyond this, the expectation was that spatially proximal communities would be more similar than spatially distant ones, based on the notion that nearby wells would be more hydrologically linked and have more similar water chemistries. Failure to find such a relationship indicates that the distance between sampling locations was greater than the scale at which the microbial communities organize. Although it is clear that the groundwater environment is heterogeneous, there are relatively few data available on the spatial and temporal scales

of variance in these systems. The results of this research imply that a great deal of variance in microbial community structure exists below the scale of measurement used (10 m) even within environments thought to be fairly homogeneous (e.g., within the aerobic zone). Indeed, the findings of Balser and Firestone (1996) and Duarte and Vaqué (1992) suggest that variance scales on the order of 10 to 100 cm may be expected, a distance less than the minimum inter-well distance at the field site. Some studies indicate microbial variance scales on the order of 1 to 3 m in cropped soils (Wollum and Cassel, 1984). However, other studies indicate a larger range of spatial scales of variability: 10 to100 m for microbiological activities in seafloor sediments (Lavigne et al., 1997), and 30 cm to 150 m in a study of spatial variation in the surface sediment of the Okefenokee swamp (Moran et al., 1987). In general, these scales appear to reflect the scales of heterogeneity in distribution of physical and chemical properties of the environment under examination. The results presented here indicate the need to consider other hydrological, physicochemical, and biological factors, besides those evaluated in this work, that might influence patch size (e.g., particulate and dissolved organic material, permeability, porosity, grain size, or substrate stability) before developing expectations about the patterns of distribution of microbiota in groundwater.

In the anaerobic zone, the results suggest two different sorts of communities may be present; the communities from the wells near the aerobic/anaerobic boundary were different from those more interior to the anaerobic zone. Given the potential for a change in water chemistry as water moves through the anaerobic zone, and the hydrologic character of the site, this is a reasonable separation. Wells along the boundary experience

a wider range of environmental conditions, both hydrological and chemical, compared to the more interior wells. For example, dissolved oxygen concentrations as high as 6.5 mg liter^{-1} have been recorded in the anaerobic boundary well PL4 following major precipitation events. However, the highest recorded concentration of dissolved oxygen for in well W2 (interior of the anaerobic region) was 1.5 mg liter^{-1} (Callaghan, 1999).

The microbial communities in the aerobic and anaerobic regions of the aquifer remained genetically distinct over time. Between summer and winter, community structure changed somewhat and these changes were similar in magnitude and direction to changes in groundwater chemistry during the same period. Communities from the aerobic wells, and the groundwater chemistry in that region, remained relatively constant at the different sampling times. In January, however, the communities in the anaerobic wells had changed significantly from earlier sampling dates; the profiles shifted so that the communities approached what is typically found in the aerobic zone. A similar change was observed in the overall pattern of groundwater chemistry for those wells. For well D1, where the change in groundwater chemistry was greatest, the microbial communities acquired some characteristics of the aerobic communities (moved through them on PC 2 (Figure 5.6)), but also some unique characteristics (which separate it from the other communities in Figure 5.6).

During the month prior to the January sampling, the research site received an unusually large amount of precipitation, and the input of this water to the aquifer may partly explain the observed changes in community structure and groundwater chemistry in the January/Winter samplings. The average monthly precipitation at the site is 73.5

mm (1997 and 1998 average) and the average winter precipitation is 98.9 mm $month^{-1}$. However, the total precipitation in January 1998 was 166.9 mm and nearly half (80.3 mm) of that fell during the week prior to sampling. Water from such precipitation events can be an important source of dissolved oxygen in shallow aquifers (Chapelle, 1993), and the percolation of oxygenated rainwater into this aquifer just prior to the January sampling could have caused the shift in the water chemistry of the anaerobic wells, making them more like the aerobic wells. Moreover, recharge events such as this may facilitate transport of dissolved nutrients into the flow system, either from the surface or as the water passes through the unsaturated zone, reducing the differences in water chemistry between the two regions of the aquifer. In contrast to the 80.3 mm of rain falling at the site in the week before sampling in January 1998, only 5.1 mm of total precipitation occurred during the 3 months prior to the August 1997 sampling. The sharp differences between the two zones of the aquifer during the summer may have reflected the absence of a recent recharge event.

The potential for precipitation events to dramatically impact this aquifer can be further illustrated by considering fluctuations in the water table levels over this same time period. For example, the water level in well W2 was 2.56 m (above mean sea level) during the August sampling and 3.38 m on the January sampling date (increase of 0.82 m (Callaghan, 1999)). Seventy percent of this increase (0.57 m) occurred during the three weeks prior to the January sampling, representing a major influx of water to the system over a relatively short time period. Further research is necessary to determine whether the sort of community turnover observed in this aquifer is a regular (i.e.,

seasonal) aspect of the system and to evaluate what level of disturbance (e.g., quantity and rate of precipitation) might be required to elicit a community response. Another factor that must be investigated is the response time - how long after an event do changes in the microbial communities become visible? Do these changes persist and, if so, for how long?

This study of groundwater microbial communities employed a relatively novel procedure (RAPD) for visualizing the overall differences between the microbial consortia at the different sampling locations. Because RAPD uses short primers of an arbitrary sequence to direct the PCR amplification, it may provide a more complete representation of the genetic structure of the entire community, compared to many of the more traditional PCR-based procedures which rely on amplification of sequences from specific organisms, groups of organisms, or genes. Monitoring the entire community as a unit, rather than gathering information on the presence/abundance of individual types of organisms, allowed for a more comprehensive comparison of the overall community dynamics and the physical and chemical conditions of the site. Major differences (both spatial and temporal) were observed within the well field and were qualitatively correlated with changes in the overall groundwater chemistry at each well. This research suggests that microbial communities in aquifers may track spatial and temporal variation in the environment to such an extent that distinct microbial communities tend to converge genetically as their environments become more similar. It remains to be seen how shifts in abundance of different microbial taxa are responsible for these changes and the spatial and temporal scale at which these changes in abundance take place.

Chapter 6.

A geostatistical analysis of small-scale spatial variability in bacterial abundance and community structure in salt-marsh creek bank sediments.

Franklin, R. B., L. K. Blum, A. C. McComb, and A. L. Mills. 2002. FEMS Microbial Ecology. 42:71-80.

Abstract

Small-scale spatial variations in bacterial abundance and community structure were examined in salt-marsh sediments from Virginia's Eastern shore. Samples were collected at 5-cm intervals (horizontally) along a 50-cm elevation gradient, over a 215-cm horizontal transect. For each sample, bacterial abundance was determined using Acridine Orange Direct Counts (AODC), and community structure was analyzed using Randomly Amplified Polymorphic DNA (RAPD) fingerprinting of whole-community DNA extracts. A geostatistical analysis was used to determine the degree of spatial autocorrelation among the samples, for each variable and each direction (horizontal and vertical). The proportion of variance in bacterial abundance that could be accounted for by the spatial model was quite high (vertical: 60 %, horizontal: 73 %); significant autocorrelation was found among samples separated by 25 cm in the vertical direction and up to 115 cm horizontally. In contrast, most of the variability in community structure was not accounted for by simply considering the spatial separation of samples (vertical: 11 %, horizontal: 22 %), and must reflect variability from other parameters (e.g., variation at other spatial scales, experimental error, or environmental heterogeneity). Microbial community patch size based upon overall similarity in community structure varied between 17 cm (vertical) and 35 cm (horizontal). Variability due to horizontal position (distance from the creek bank) was much smaller than that due to vertical position (elevation) for both community properties assayed. This suggests that processes more correlated with elevation (e.g., drainage and redox potential) vary at a smaller scale (therefore producing smaller patch sizes) than processes controlled by distance from the creek bank.

6.1. Introduction

Although individual bacterial cells function at a scale that befits their small size (Brock, 1987), their combined localized activities mediate processes that are important at the landscape scale. It is at this scale that microbial reactions are most often studied, and a great deal of effort is currently being expended to try to relate the structure of these communities to observed functional phenomena. When studying microorganisms, the boundaries used to define a community are generally utilitarian and dictated by the required sample size, the researcher's perception of environmental variability, and the overall scale of the property of interest. As a result, measurements of microbial community attributes typically represent broad-scale characterizations and rarely consider the small spatial scale at which individuals and populations may actually be interacting.

Though the broad-scale approach to studying microbial communities has been useful for monitoring large-scale changes in microbial dynamics, studies of microbial community patch size are rare, and knowledge about the scales at which microbial interactions and associations become important is incomplete. The total capacity of microbial communities in the environment is the sum of the activity of several "unit" communities of microorganisms (Swift, 1984), in distinct microhabitats, whose separate activities are pooled into what scientists observe as ecosystem function. In order to understand well how these spatial units fit together and how the activity of the distinct units contributes to overall ecosystem function, a better understanding of the distribution of microorganisms (and microbial communities) in space is needed.

In sampling theory, spatial scale is defined by several characteristic properties: grain size, sampling interval, and extent (Legendre and Legendre, 1998). *Grain size* is the size of the elementary sampling units (e.g., the volume of sample), and defines the resolution of the study (Schneider, 1994). *Sampling interval* is the average distance between sampling units, and the *extent* is the total area included in the study. Depending on the ecological question being addressed, and what is already known about the scale of the process of interest, the dimensions of these components vary. For a given sampling design, no structure can be detected that is smaller than the grain size or larger than the extent of the study; in this way, the sampling design defines the observational window for spatial pattern analysis (Legendre and Legendre, 1998).

Previously, researchers have considered the spatial distribution of microorganisms in many different environments and at a variety of spatial scales. In ecological studies, the grain size is generally too large to permit analysis of the location or activity of individual organisms, though experiments have been conducted at the microscale to examine these properties (Bockelmann et al., 2002; Dandurand et al., 1995; Guggenheim et al., 2001; Manz et al., 2000; Nunan, et al., 2001). For example, electron microscopy has been used to study the rhizosphere colonization patterns of *Pseudomonas flurorescens* using a sampling interval of 5 μm (Dandurand et al., 1997). At larger scales, studies in agricultural soils have demonstrated that significant spatial heterogeneity may exist for microbiological processes (Bending et al., 2001; Grundmann and Debouzie, 2000), community structure (Balser and Firestone, 1996; Cavigelli et al., 1995), and abundance (Nunan et al., 2001; Wollum and Cassel, 1984); patch size estimates range widely from as small as 2 mm (Grundmann and Debouzie, 2000) to nearly 10 m

(Franklin and Mills, 2003). Similar studies have been conducted in grassland and forest soils (Both et al., 1992; Kuperman et al., 1998; Morris, 1999; Ritz et al., 2001; Robertson et al., 1988; Saetre and Bååth, 2000), in a shallow coastal aquifer (Franklin et al., 1999b), and in the open ocean (Duarte and Vaqué, 1992; Mackas, 1984). For salt-marsh and marine sediments, variation has been examined at small scales (i.e., < 1 m^2 (Berardesco et al., 1998; Danovaro et al., 2001; Scala and Kerkhof, 2000)), and at intermediate (< 150 m (Moran et al., 1987)) and larger distances (e.g., km (Scala and Kerkhof, 2000)). In general, all of these studies reveal that microbial communities can be organized at a variety of spatial scales, which likely reflect the scales of heterogeneity in the distribution of physical and chemical properties for the environment under investigation.

In addition to the theoretical implications that motivate research about the spatial distribution of microorganisms, the results of such studies have important practical applications for scientists designing and planning experiments at the field and landscape scales. While many ecological theories and models acknowledge that elements that are close to one another in space or time are more likely to be influenced by the same generating processes, the same energy inputs, or a similar physical environment, the classical statistical procedures employed to analyze these phenomena assume independence of observations. Statisticians generally count one degree of freedom for each independent observation, which allows them to choose an appropriate statistical distribution for testing; the lack of independence that arises from the presence of autocorrelation makes it difficult (in many cases, impossible) to accurately determine the number of degrees of freedom and correctly perform tests such as correlation, regression, or analysis of variance. Positive autocorrelation reduces within-group variability,

artificially increasing the amount of among-group variance, and often leads to the determination that differences among groups are significant, when in fact they are not (Legendre et al., 1990).

Some procedures exist that allow researchers to make corrections and perform statistical analyses in the presence of spatial autocorrelation; for an overview, see: Cliff and Ord (1981), Legendre et al. (1990), Legendre (1993), Legendre and Legendre (1998), and Oberrath and Bohning-Gaese (2001). However, the application of these techniques is often limited by constraints such as sample size or the physical distribution of sampling locations (e.g., a procedure may require sampling locations to be regularly spaced along a grid (Legendre et al., 1990)). Another solution is to design a sample collection scheme so that there is little spatial structure present in the data, and then use parametric statistical hypothesis tests. In this case, samples must be collected close enough together that they represent replicates of the system under investigation, but they must be placed far enough apart to avoid autocorrelation. Regardless of which approach one chooses (correction of statistical procedures or modification of experimental design), it is first necessary to describe the type of autocorrelation present in a variable (e.g., gradient vs. patches) and estimate its extent. There are several procedures available to test for the presence of spatial structure in ecological data (for reviews, see: Goovaerts (1998), Legendre (1993), Legendre and Fortin (1989), Robertson (1987), and Rossi et al. (1992)); geostatistics is one powerful tool that can provide insight into spatial structure and quantitatively describe spatial variation by expressing a measure of association, or autocorrelation, between two samples as a function of the distance between them. Geostatistical analyses are commonly used in soil and mineral science, but less so in microbial ecology

(Castrignanò et al., 2000; Dobermann et al., 1995; Franklin and Mills, 2003; Grundmann and Debouzie, 2000; Kuperman et al., 1998; Mackas, 1984; Morris, 1999; Murray, 2001; Ritz et al., 2001; Schlesinger et al., 1996).

The purpose of the present study was to examine the spatial structure of microbial communities in salt-marsh sediments using traditional geostatistical techniques. Sampling of unvegetated creek bank sediment in a *Spartina alterniflora* dominated salt marsh was done at 5-cm intervals; the community in the samples was characterized microscopically (for total concentration of bacterial cells) and with DNA fingerprinting (to determine the overall genetic similarity between communities). These data were then analyzed to determine microbial community patch size, the amount of spatial autocorrelation among the samples, and the relative importance of horizontal (distance from creek bank) versus vertical (elevation) separation of communities. Each of the analyses performed confirmed that spatial autocorrelation existed at a relatively small-scale (10 – 100 cm). In general, spatial structure in abundance was organized with a correlation length scale larger than that for community structure, and the patch size for the communities was greater in the horizontal direction than in the vertical. These results suggest that the study of salt marshes at spatial scales such as these could provide insight into structuring and distribution of microbial communities in these systems, and help scientists to understand within-marsh biogeochemical process variation. Moreover, the work has important implications for researchers conducting field experiments as it indicates that sampling of these sediments at spacings less than the correlation length scale of the property of interest may result in inaccurate statistical analyses and incorrect conclusions.

6.2. Materials and methods

6.2.1. Sample collection

Sediment samples were collected from Phillips Creek marsh in the Virginia Coast Reserve Long Term Ecological Research site (VCR-LTER), on Virginia's Eastern Shore, during September of 1999. The area sampled (37° 27.496´N, 75º 50.075´W) was an unvegetated, intertidal portion of the bank of a minor tributary of Phillips Creek. For each sample, a small core was taken from the surface of the marsh to a depth of approximately 5 cm, using de-tipped, 10-mL plastic syringes. The 44 sampling locations in this study ranged vertically over 50 cm of elevation; position was measured to the nearest 0.25 cm. The samples were not regularly spaced over the elevation gradient, but the average separation between any pair of adjacent samples was 1.5 cm. The sampling locations were regularly spaced at 5-cm intervals in the horizontal direction, ranging over a length of 215 cm (Figure 6.1).

After collection, the sediment samples were transported to the lab on dry ice, and stored at -20°C until Acridine Orange direct counts (AODC) (Bottomley, 1994; Hobbie et al., 1977) and DNA extractions could be performed.

6.2.2. DNA extraction and quantification

Microbial community DNA was extracted from the sediment samples using the MoBio UltraClean Soil DNA isolation Kit (Solana Beach, CA); the amount of sediment used in each extraction ranged from 0.25 g to 0.83 g. The amount of sediment used in each extraction was varied, based upon cell counts (AODC), so that the concentration of

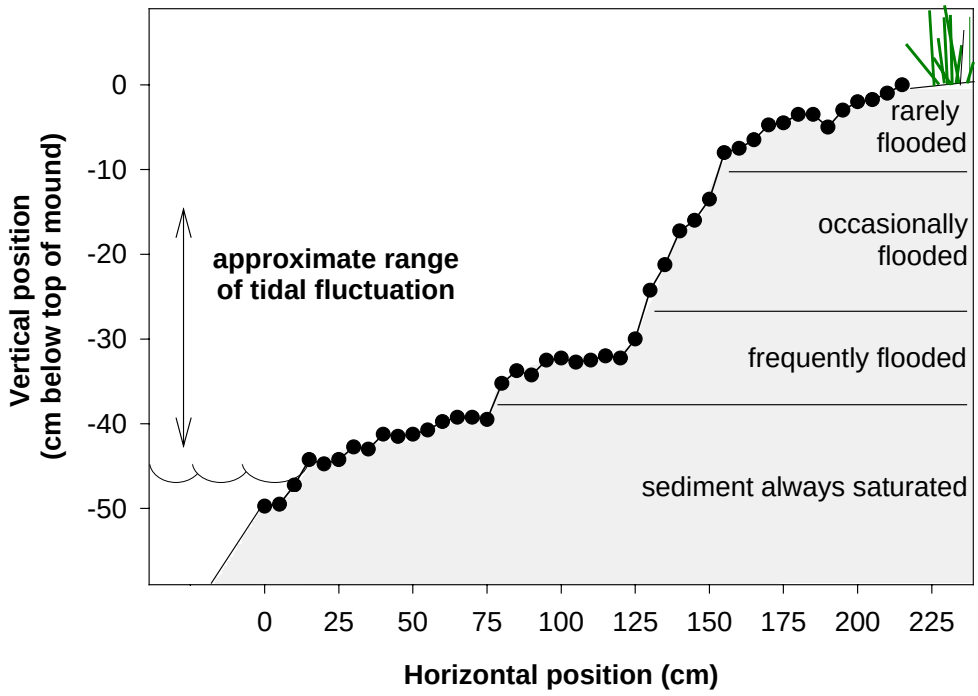

Figure 6.1. Map of creek bank showing sampling locations (indicated with •), position of Phillips Creek, and approximate range of tidal influence along the slope. Sampling locations were divided into four groups based on their flooding frequency and elevation.

DNA obtained from each sample would be approximately equal. Cells were lysed using the vortex procedure described in the kit documentation. Purified DNA was resuspended in 10 mM Tris buffer and stored at -20°C. The concentration of DNA from each sample was measured using the PicoGreen dsDNA quantification kit (Molecular Probes, Eugene OR).

6.2.3. RAPD community fingerprinting

6.2.3.1. RAPD conditions

Randomly Amplified Polymorphic DNA (RAPD) reactions were performed as previously described (Franklin et al., 1999a). Briefly, a 5-µl portion of a DNA solution (containing approximately 350 pg DNA) was added to a 20-µl reaction mixture containing: 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM primer (Operon Technologies, Alameda, CA), and 1 unit of *Taq* DNA polymerase. The PCR program consisted of 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. Amplification products were separated by electrophoresis in 1.75 % agarose gels (premixed with ethidium bromide (0.25 µg ml^{-1})), and photographed under UV light. Each sample was amplified several times, each time with a different RAPD primer (see below), and the results pooled to represent a single community fingerprint.

6.2.3.2. Primer selection

Previous work, characterizing the pelagic microbial communities in Phillips Creek (Franklin et al., 1999a), was used to guide primer selection in this study. Each primer successfully used in the earlier study was tested on a subset of the sediment samples; criteria such as number of bands produced, clarity and distinctness of bands, and reproducibility of the RAPD fingerprints were used to select the best primers to profile the entire set of samples. Primers C4, F1, F3, F4, F5, F7, and T7 were chosen (sequences available in Franklin et al. (1999a) and from Operon Biotechnologies (www.operon.com)). These primers are short (10 bp), random sequences that can anneal at numerous locations throughout a genome, and they are not selective for specific organisms, groups of organisms, or genes. Because of this, RAPD can detect sequence variability that is distributed throughout the microbial DNA pool, theoretically producing a fingerprint of the composite genetic composition of the entire community.

6.2.3.3. RAPD data analysis

After agarose-gel electrophoresis, the raw data from RAPD consist of a series of bands, each corresponding to a certain size DNA fragment. For each primer, each amplification band was treated as a unit character and scored as present or absent in each sample (i.e., recorded as 1 or 0). The results for all of the primers were then pooled into a single large dataset. Collectively, these primers produced a total of 87 bands and individual samples contained between 11 and 34 bands. The average number of bands produced for a given sample was 21.

6.2.4. Variogram analysis of spatial autocorrelation

In this study, the variation in bacterial abundance (as measured by AODC) and in community structural similarity (as determined by RAPD fingerprinting) was compared using a geostatistical semi-variogram analysis to study autocorrelation as a function of distance. The correlation between spatial separation and each community parameter was modeled considering the two distance components (horizontal and vertical) separately, rather than using the Euclidian distance between each set of samples, as it was not expected that the scale of variability in the horizontal and vertical directions would be the same. Moreover, as the samples were taken along a slope, the vector connecting many of the points (using Euclidian distance) passed through the air, rather than the sediment, so it did not seem reasonable to use this separation distance to model the spatial autocorrelation of the microbial communities. Comparing each sampling location with each of the other 43 resulted in a total of 946 points to analyze (this is the number of points in the upper (or lower) triangle matrix between each sampling location).

Prior to constructing a variogram, it was necessary to segregate the data into distance classes (bins). The purpose of binning the data was to obtain the best resolution (maximum detail) at small distances without being misled by structural artifacts due to whatever particular size class was chosen. To determine the appropriate number of bins for each analysis, Sturge's rule (Legendre and Legendre, 1998) was used, which states that the appropriate number of classes = $1 + 3.3 \log_{10} m$, where m is the number of points in either the upper or lower triangle matrix (in this case, 946); the calculation suggests that 10 size classes are appropriate for this dataset. Furthermore, variograms are

generally not valid beyond 1/2 of the maximum distance between samples, and so the appropriate lag distance (the distance increment for each class) was calculated as the maximum pair distance divided by 2, and then subdivided into 10 equal classes (Englund and Sparks, 1991). For the vertical direction, this resulted in a 2.5-cm lag distance; for the horizontal direction, a lag distance of 10 cm was established.

To calculate a variance term for the AODC, each value was first normalized by dividing it by the overall average concentration of cells among all samples. The inverted covariance was then calculated between each pair of samples using GEOEAS (Englund and Sparks, 1991). A traditional variance term could not be calculated for the RAPD results (multivariate binary data); instead, the relative similarity between each pair of samples was determined using the Jaccard coefficient (which is based upon the proportion of positive bands shared by each sample pair (Sneath and Sokal, 1973)). This similarity matrix was then converted to a dissimilarity matrix (Dissimilarity = 1 - Similarity), which represents the relative difference in community genetic structure between each pair of samples. Plots of relative dissimilarity versus spatial separation distance should take the form of a typical variogram.

Experimental variograms are often fit with a continuous function, to smooth out sample fluctuations and estimate useful model parameters (e.g., the correlation length scale (range) and the spatial dependence). Several types of functions are available including the linear, Gaussian, exponential, and spherical models. A linear variogram would indicate a linear spatial gradient, and would represent a situation where the samples are spatially autocorrelated at all distances measured (the sampling distance is

not large enough to capture all of the spatial variability at that scale). Gaussian, exponential, and spherical models are bounded in that they level off, either at a given range value (spherical model) or asymptotically (exponential and Gaussian models). Differences between these functions lie mostly in the shape of the left hand portion of the curves, near the origin; in practice, the spherical and exponential models do not differ much (Legendre and Legendre, 1998). Several authors have warned against the risk of numerical instability associated with the Gaussian model, and it is rarely used at this point (Goovaerts, 1998).

For each parameter (community structure and AODC), separate variograms were constructed for each direction (horizontal and vertical). Data were then fit with an exponential model, as suggested by Legendre and Legendre (1998):

$$y = C_0 + C_1[1 - \exp(-3\frac{x}{a})]$$

where y is the variance term (either inverted covariance (for AODC) or dissimilarity (for genetic community structure)), and x is the spatial separation distance. From the model, C_0, C_1, and a were estimated; C_0 is a parameter quantifying the nugget effect (the amount of variability at distance = 0), C_1 is a spatially structured component of the model, and a is the range (the distance beyond which variance is no longer a function of spatial separation). The sill (C) is the y value at which the variogram levels off, and was calculated as: $C = C_0 + C_1$.

One difficulty with using equal distance classes when constructing a variogram is that the number of pairs of points in large distance classes is often too small for valid testing; to avoid this problem, only the spatial structure of the first two thirds of the variogram was modeled (Legendre and Fortin, 1989). All regressions were performed in SigmaPlot (Version 4.0), and R^2 was used to measure the goodness of fit of the model to the data.

6.2.5. Determination of group differences

6.2.5.1. Community structure

In addition to the geostatistical analyses, the data were also analyzed to determine if any group differences existed between samples positioned along the elevation gradient. Samples were divided into four groups (Figure 6.1), based on elevation, horizontal position, and frequency of inundation. The four groups were (1) sediment always saturated, samples 1 – 16; (2) sediment frequently flooded, samples 17 – 26; (3) sediment occasionally flooded, samples 27 – 31; and (4) sediment rarely flooded, 32 – 44. A Mantel test (Mantel, 1967; Sokal, 1979) was used to evaluate whether overall microbial community structure was significantly different among the various groups. In general, a Mantel test determines the amount of correlation between two matrices, and a permutation procedure is used to assess the significance of this correlation. In this application, the two matrices being compared were (1) the observed dissimilarity matrix calculated from the RAPD data, and (2) a conceptual model matrix that defined group identity. Data were analyzed to test the null hypothesis that there was no difference in within and between-group genetic similarities of the communities using the Mantel-Struct

program (Miller, 1999). A Monte Carlo procedure (with 5000 permutations) was then used to control for the impact of spatial autocorrelation among the sampling locations and to evaluate the significance of the group differences.

6.2.5.2. Bacterial abundance (AODC)

A Mantel's test was also used to compare bacterial abundance in the different elevation groups. First, a dissimilarity matrix was calculated by determining the absolute value of the different between each sample pair, divided by the maximum difference for all pairs. This matrix, along with the group identity for each sample, was analyzed using the Mantel-Struct program (Miller, 1999), as described above.

6.3. Results

6.3.1. Variogram analysis of spatial autocorrelation

Figure 6.2 shows the results of the geostatistical analyses for each parameter (bacterial abundance and community structure) for each direction (horizontal and vertical). The data were fit with an exponential model, and the model output is presented in Table 6.1. In general, the model fit the data quite well (Table 6.1, Figure 6.2); R^2 varied between 0.62 and 0.96, and all correlations were significant, with $p < 0.005$. For AODC, the spatial dependence (the proportion of variance in the data that was accounted for by the spatial model) was quite high. In the horizontal direction, the spatial model could account for 73 % of the variance in microbial abundance; for the vertical direction, 60 % of the variation in bacterial abundance could be correlated with the spatial

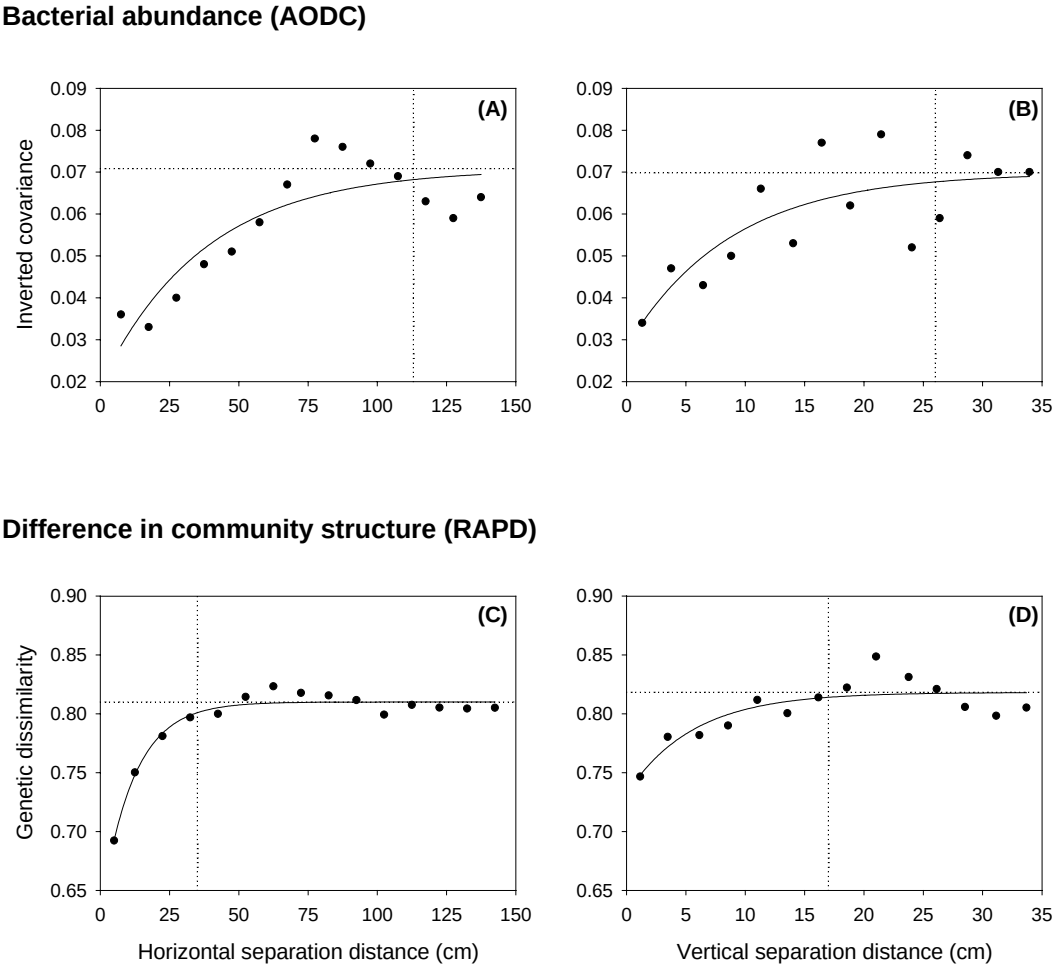

Figure 6.2. Variograms constructed to model spatial autocorrelation in bacterial abundance (A and B) and difference in microbial community structure (C and D) along the creek bank. Directional variograms were constructed for each parameter based upon either the horizontal (A and C) or vertical (B and D) separation of the samples. Best-fit lines, constructed using an exponential model, are presented, as are the sill (asymptotic value of the curve) and range (separation distance where Y is 95% of the sill).

Table 6.1. Summary of model parameters obtained from fitting an exponential equation to the experimental variograms.

Parameter and direction	Nugget (C_0)	Sill ($C=C_0 + C_1$)	Nugget effect (C_0/C)	Spatial dependence (C_1/C)	R^2	Range (cm)
Bacterial abundance (AODC):						
Horizontal	0.019	0.071	0.27	0.73	0.76	113
Vertical	0.028	0.070	0.40	0.60	0.62	26
Community structure (RAPD):						
Horizontal	0.631	0.810	0.78	0.22	0.96	35
Vertical	0.732	0.818	0.89	0.11	0.79	17

separation of samples. The range (the distance beyond which data exhibit no spatial autocorrelation) was 26 cm in the vertical direction and 113 cm in the horizontal. The sill (the value of the variogram at distances beyond the range) was the same for both directions.

In geostatistics, anisotropy is said to be present in data when the autocorrelation function is not the same for all geographic directions. In the case of bacterial abundance, the analyses produced the same sill for the two different directions, but different ranges; this phenomenon is referred to as geometric anisotropy (Isaaks and Srivastava, 1989). A geometric anisotropy ratio can be calculated as the ratio of the larger range to the smaller range; here that ratio is 4.3 (horizontal / vertical). The means that, on average, the same amount of variability occurred over 4 horizontal distance units as occurred in 1 vertical distance unit. It is interesting to note the similarity in this value compared with the aspect ratio (horizontal distance / vertical distance = 4.3).

For similarity in community structure, spatial dependence was lower (horizontal: 22 %, vertical: 11 %), and most of the variation in community structure was not accounted for simply by considering the spatial separation of the samples. The range of spatial extent for overall community structural similarity was smaller than was calculated for bacterial abundance: 35 cm for the horizontal direction and 17 cm for the vertical. As with bacterial abundance, the sills for the two directions were similar and a geometric anisotropy ratio could be calculated. In this case, the anisoptropy was 2 – smaller, but in the same direction as for bacterial abundance (horizontal / vertical).

6.3.2. Analysis of group differences

An analysis of group differences was performed to evaluate whether the different flooding zones contained communities whose structure and abundance were significantly different. A Mantel test was performed, and a Monte Carlo procedure (using 5000 permutations) was employed to assess the significance of the test statistic. A Bonferoni type correction was used to adjust the α level, depending on the number of comparisons made (Legendre and Fortin, 1989); a modified α level of 0.008 ($\alpha = 0.05$ divided by 6 comparisons) was used.

Overall, the sample groups were found to contain significantly different microbial communities, as assayed by RAPD fingerprinting ($p = 0.0002$). More specifically, group 4 (rarely flooded/high marsh) was found to differ from all of the other groups, and group 3 was significantly different from group 1 (Table 6.2).

For microbial abundance, the average concentration of cells ($\times 10^{10}$ cells ml^{-1} sediment) was: group 1 - 7.4, group 2 - 5.6, group 3 - 6.8, and group 4 - 7.8. Overall, these differences were significant ($p = 0.03$), with group 4 (rarely flooded/high marsh) being most distinct (Table 6.2).

6.4. Discussion

The overall goal of this study was to quantify spatial autocorrelation among the microbial communities residing in salt-marsh creek bank sediments. In particular, we sought to determine the extent to which the spatial distribution of samples may contribute to overall between-sample variability, and to estimate microbial community patch size.

Table 6.2. Results (*p* values) from a series of Mantel tests comparing community properties for the different elevation groups.

Elevation group	Bacterial Abundance			Community structure		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
1 – Always saturated	–			–		
2 – Freq. flooded	0.62	–		0.088	–	
3 – Occasionally flooded	0.99	0.007*	–	0.0008*	0.018	–
4 – Rarely flooded	0.003*	0.0008*	0.39	0.004*	0.0002*	0.0006*

* Significant comparisons, after α was adjusted to 0.008 (see text).

In general, the results demonstrated that small-scale variability exits, both in terms of total bacterial abundance (AODC) and in terms of community structure (RAPD DNA fingerprinting results). The "patch size" of the microbial communities found in these sediments varied somewhat, depending on the parameter used to assay the organisms and the way that the spatial separation of the sampling units was calculated; patch size estimates ranged between approximately 10 and 100 cm. These values are similar to those obtained by other researchers, in other environments, as outlined in the Introduction.

The correlation length scales for bacterial abundance (AODC) were larger than the values obtained when considering community structure, for both directions (vertical: 113 cm vs. 35 cm; horizontal: 26 cm vs. 17 cm). This finding suggests that the environmental factors controlling these two community attributes may be different, and may vary at different spatial scales in the environment. Alternately, these results could be due a single environmental parameter influencing the two microbial attributes differently or to a different extent. Simultaneous measurement of the distribution of microbial communities and physicochemical properties (e.g., organic matter concentration, sediment moisture content, or redox status) at small spatial scales would add a great deal to our understanding of how environmental heterogeneity can influence microbial community development, and, conversely, how microbial communities may alter their microenvironment. Unfortunately, the samples collected for this study were too small to permit measurement of the microbial communities as well as any physicochemical parameters.

Most of the studies of microbial community variability at small spatial scales have considered more general properties (e.g., total abundance, biomass, or activity (Duarte and Vaqué, 1992; Moran et al., 1987; Morris, 1999)), while relatively few studies have examined the distribution of microbial community structure (Balser and Firestone, 1996; Franklin et al., 1999b; Mackas, 1984; Saetre and Bååth, 2000). Of those that have, the range over which the communities are spatially autocorrelated is generally smaller than the range established for more broad microbial community properties. However, there are very few studies that have compared these two aspects of the same community (Both et al., 1992; Mackas, 1984). Likewise, studies that have examined the distribution of guilds (e.g., denitrifiers (Scala and Kerkhof, 2000)) or specific groups of organisms (Cavigelli et al., 1995; Grundmann and Debouzie, 2000; Saetre and Bååth, 2000) in the environment tend to find smaller correlation length scales compared to those studies that assay for more general microbial parameters. Part of this discrepancy may be due to the fact that researchers whose specialization involves studying specific functions or particular groups of organisms are more likely to conduct experiments at small spatial scales, while researchers who study more general parameters often collect larger samples, which can limit the resolution of a study.

In this study, the spatial dependence (the percent of the total variance in the data that can be explained by considering the spatial separation of the sampling units) was much less for the analyses that considered community structure (horizontal: 22 %, vertical: 11 %) than for the analyses that considered bacterial abundance (horizontal: 73%, vertical: 60 %). Most of the variability in community structure must come from variation at other scales, experimental error, or the influence of other environmental

parameters. The reproducibility of the RAPD procedure is sensitive to a number of experimental factors (Davin-Regli et al., 1995; Meunier and Grimont, 1993), and experimental error might partially explain the relatively large nugget effect observed in the geostatistical analyses of the RAPD data. However, recent work has shown the repeatability of RAPD fingerprinting to be quite good with microbial community DNA samples (Franklin et al., 1999a; Wikström et al., 1999), and it is unlikely that methodological problems contributed significantly to the results presented here.

Given the relatively small portion of the variance in community structure that was spatially dependent, it seemed reasonable to analyze the RAPD data to see if there were any differences between groups of samples located at different elevations (Figure 6.1). Groups were defined based on vertical position and frequency of inundation, and roughly correspond to four areas between low and high marsh. The RAPD profiles obtained for the group 4 samples (rarely flooded/high marsh) were significantly different from those obtained in any of the other zones (Table 6.2). Similarly, the communities inhabiting group 3 (occasionally flooded) were significantly different from the group 1 (saturated sediment/low marsh) communities. For bacterial abundance (AODC), the group differences were not as strong, though group 4 was, again, distinct from the other samples. The flooding regime along the creek bank could influence the microbial communities in a number of ways. Besides the direct effect of inundation on microbial community structure (e.g., flooding could add or remove organism types), there are a number of different environmental parameters (e.g., sediment moisture content, redox

status) that may covary in response to the patterns of water movement; further study would be necessary to establish whether any of these parameters are important in generating the community differences observed here.

One of the main problems when working with samples that are spatially autocorrelated is that the use of parametric statistical procedures for data analysis is not appropriate. As part of this study, we wanted to estimate the range of spatial influence of microbial abundance within these sediments, and then to determine whether sampling beyond this range provides a different estimate of mean bacterial concentration, compared to sampling that was conducted at separation distances smaller than the correlation length scale. To test whether the estimate of abundance one would obtain by sampling two spatially autocorrelated locations was significantly different from the value one would obtain by sampling two locations that were spatially independent, we calculated the average AODC between each pair of samples (to simulate several sampling efforts), and then categorized each average as having been obtained using spatially autocorrelated samples or as being spatially independent. After sorting the averages into these two groups, an ANOVA was used to determine whether the estimates of abundance obtained using spatially autocorrelated samples were significantly different from that obtained using pairs of independent samples. Overall, the estimate of mean AODC was significantly lower using spatially autocorrelated samples ($6.9 \times 10^{10} \pm 4.2 \times 10^{8}$ (st. error)) compared to spatially independent samples ($7.6 \times 10^{10} \pm 5.8 \times 10^{8}$ (st. error)) (ANOVA results: d.f. = 945, F = 88.96, $p < 0.0009$). This exercise demonstrates the important impact spatial autocorrelation may have on a scientist's estimate of mean environmental properties in a given region or habitat.

Another major trend revealed in this analysis is the anisotropy associated with the distribution of microorganisms in the sediments. For both community parameters, the patch size was always greater when horizontal separation was used as the distance measure, compared to vertical separation. Partly, this finding could be an artifact of the sampling design – our sample density was much higher for X direction than for the Y direction (44 samples / 50-cm vertical elevation, versus 44 samples / 215-cm horizontal expanse). The higher sampling density means that there was a smaller average separation distance considering elevation (1.5 cm) compared to the horizontal direction (5 cm), and this smaller separation distance increases the opportunity to detect smaller-scale variability. Since microbial communities are organized at a hierarchy of spatial scales, it is possible that the sample design used here simply captured community variation at two different levels. It is also possible that there are different environmental parameters influencing community development in the two directions, and that the processes more correlated with elevation (e.g., drainage and redox potential) vary at a smaller scale than the processes controlled by distance from the creek bank. It is important to note that, throughout most of this discussion, the horizontal and vertical components of space have been discussed as being independent, though they are not. For example, one might expect drainage and moisture content of two samples located the same distance from the creek bank, but at different elevations, to be different.

Within marsh ecosystems, microbial communities serve many critical functions, including the decomposition of organic material and the biogeochemical cycling of minerals and nutrients. Researchers generally see a great deal of variation in microbial community structure and processes, both within and between marshes. Often times,

biogeochemical process variation within visibly homogenous environments of a single marsh is greater than among marsh variation (Frischer et al., 2000; Hackney and De La Cruz, 1980; Hackney and De La Cruz, 1986; Hines et al., 1999). The results presented here suggest that community structure and microbial abundance can vary at small scales (< 1 m) in these systems, and that this information needs to be incorporated into the experimental design when sampling these habitats. It is reasonable to expect the patch size of the microbial community to vary in different environments, and some care must be taken when trying to extrapolate the results of this work to other systems.

Chapter 7.

Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field.

Franklin, R. B. and A. L. Mills. 2003. FEMS Microbial Ecology. 44:335-346.

Abstract

To better understand the distribution of soil microbial communities at multiple spatial scales, a survey was conducted to examine the spatial organization of community structure in a wheat field in Eastern Virginia (USA). Nearly 200 soil samples were collected at a variety of separation distances ranging from 2.5 cm to 11 m. Whole-community DNA was extracted from each sample, and community structure was compared using amplified fragment length polymorphism (AFLP) DNA fingerprinting. Relative similarity was calculated between each pair of samples and compared using geostatistical variogram analysis to study autocorrelation as a function of separation distance. Spatial autocorrelation was found at scales ranging from 30 cm to more than 6 m, depending on the sampling extent considered. In some locations, up to four different correlation length scales were detected. The presence of nested scales of variability suggests that the environmental factors regulating the development of the communities in this soil may operate at different scales. Kriging was used to generate maps of the spatial organization of communities across the plot, and the results demonstrated that bacterial distributions can be highly structured, even within a habitat that appears relatively homogeneous at the plot and field scale. Different subsets of the microbial community were distributed differently across the plot, and this is thought to be due to the variable response of individual populations to spatial heterogeneity associated with soil properties.

7.1. Introduction

Microorganisms are not distributed uniformly in the environment, rather their abundance and activity change along environmental gradients. Even within a homogeneous system, biological processes (e.g., growth or colony formation) may produce aggregations of organisms at various spatial scales. Soil systems are particularly heterogeneous, and this heterogeneity arises as a result of the interaction of a hierarchical series of interrelated variables that fluctuate at many different spatial and temporal scales. The factors that affect microbial survival and community structure in soils are known to be both biotic (e.g., predation and competition) and abiotic (e.g., temperature or pH). Some of these processes are primarily important at microscopic scales (e.g., particle size and pore space structure), whereas others act over larger distances (e.g., vegetation cover and precipitation). These soil properties do not vary independently; rather, the general perception is that any such variable measured at a certain point in space and time is the outcome of several physical, chemical, and biological processes, all of which are spatially variable.

Given that environmental factors do not necessarily operate independently, or at distinct spatial scales, studying microbial systems using a single analytical scale cannot provide a complete understanding of community dynamics. Multi-scale comparisons, in which patterns are analyzed at several different spatial scales, may be more useful when trying to identify the factors that control community development. Conclusions about the organization of microbial communities, the effect of disturbance, or the roles of various limiting factors are likely to differ at different spatial scales (Wiens et al., 1986). Moreover, the characterization of microbial communities at several different scales may

help explain paradoxes that arise when different investigators, studying similar communities but at different scales, arrive at different conclusions about the factors that structure those communities. These disagreements may reflect viewpoints of different scales, and not differences in the way communities are organized (Rahel, 1990).

Previous work studying spatial organization in soil microbial systems has primarily focused on the distribution of individual cells (Dandurand et al., 1995; Fendorf et al., 1997; Nunan et al., 2001; Nunan et al., 2002), specific types of organisms (Bending et al., 2001; Both et al., 1992; Dandurand et al., 1997; Felske and Akkermans, 1998; Grundmann and Debouzie, 2000), or collective parameters such as bacterial abundance or total biomass (Franklin et al., 2002; Kuperman et al., 1998; Morris, 1999; Robertson et al., 1997; Saetre, 1999; Smith et al., 1994). There are fewer studies that have considered variations in community structure (Acosta and Lynn, 2002; Cavigelli et al., 1995; Franklin et al., 2002; Ranjard et al., 2000; Saetre and Bååth, 2000) or function/activity (Gorres et al., 1998; Parkin et al., 1987; Robertson et al., 1988; Saetre, 1999). In general, these studies have concentrated on understanding spatial variability at a single analytical scale, and have found significant spatial autocorrelation at a variety of separation distances, ranging from μm to km, depending on the spatial extent studied. Recently, scientists have begun to focus on multi-scale comparisons, and have found evidence for nested scales of spatial structure (Bruckner et al., 1999; Ettema and Wardle, 2002; Robertson and Gross, 1994; Saetre and Bååth, 2000; Stenger et al., 2002). For example, Nunan et al. (2002) studied the spatial distribution of soil bacteria at three different scales, ranging from μm to meters, and found that the distribution of individual bacterial cells was organized at two scales in the subsoil, and at a single scale in the topsoil.

Studies conducted in agricultural and shrub-steppe ecosystems suggest that microbial biomass and activity may be spatially dependent at scales less than 1 m, nested within a larger scale related to variations at the landscape level (Robertson et al., 1997; Ronimus et al., 1997; Smith et al., 1994). The presence of nested scales of variation suggest that the various factors regulating the development of microbial communities in the soil ecosystems may operate at different scales (Robertson and Gross, 1994), and a simultaneous analysis of the multi-scale spatial variability of microbial community structure and soil microenvironment could help identify these factors and determine their relative influence.

The present study was designed to address the general need for increased research into multi-scale patterns of spatial organization in soil systems. In particular, the research focused on quantifying the spatial patterns associated with microbial community structure at the cm to meter scale using geostatistical techniques. Nested levels of spatial autocorrelation were observed (ranging from 30 cm to more than 6 m), and, in some locations, up to four distinct ranges of spatial influence were quantified.

7.2. Materials and methods

7.2.1. Site description and sample collection

Soil samples were collected from an agricultural field on the eastern shore of Virginia (USA) in May 2000 (37° 17.62´N, 75º 55.53´W). The field was planted with durum wheat (*Triticum turgidum*), and the crop was approximately 75 days old on the day of sampling. Samples were collected with separation distances ranging from 2.5 cm to 11 m, using the sampling scheme detailed below. At each sampling location, the loose

layer of surface material was removed, and a small hole (1.5 cm diameter) was dug to collect 5 – 10 grams of soil. The samples were placed on ice for transport to the lab, where they were sifted (approximately 750-μm mesh size), to remove gravel, plant, and root material, and stored at -80°C.

7.2.2. Sampling scheme

The basic sampling design was a square with 7.1 m edges and 10 m diagonals (Figure 7.1). Samples were collected at regular intervals around the perimeter of the block (1.8 m separation distance), and at 1 m intervals along the diagonals. At each node (A, B, C, D, and X), more concentrated sampling efforts were employed.

Nested within the original sampling grid, a second set of samples were collected at 10-cm increments in a cross shape surrounding each node. Five samples were collected in each direction - north, south, east, and west - from the center node. Nested within this area, a third set of samples was collected at 2.5-cm increments around each node, following the same pattern (2.5, 5.0, 7.5, and 10 cm in each direction). A total of 193 soil samples were collected, 33 at each node and 28 at larger separation distances.

7.2.3. DNA extraction and quantification

Whole-community DNA was extracted from 0.25-gram subsamples of soil with the MoBio Soil DNA isolation kit (Solana Beach, CA) using the alternative heat shock lysis procedure described in the kit documentation. Purified DNA was resuspended in 10 mM Tris buffer and stored at –20°C. The concentration of DNA in each sample was determined using the PicoGreen reagent (Molecular Probes, Eugene, OR).

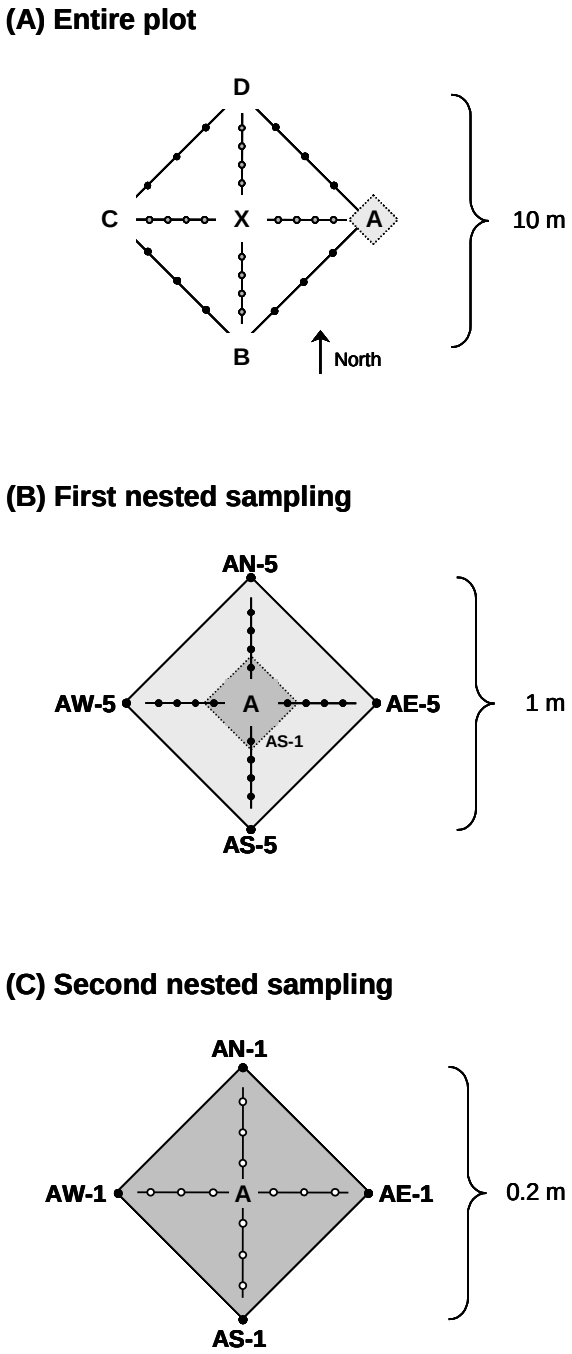

Figure 7.1. Map of the sampling scheme. (A) The sampling area was a 50 m^2 square (diamond) with 10-m diagonals. Around the perimeter of the square, samples were collected at 1.8 m increments, and at 1 m increments along the diagonals. At each node (A – X), more concentrated sampling efforts were employed using a nested sampling pattern. Node A is presented in the figure as an example. Additional samples were collected at 10 cm increments (B) and 2.5 cm increments (C) in a cross shape surrounding each node

7.2.4. AFLP

Amplified fragment length polymorphism (AFLP) analysis was performed using the Perkin Elmer Microbial Fingerprinting Kit (PE Applied Biosystems, Foster City, CA). For community analysis, the manufacturer's instructions for analysis of individual bacterial strains were modified as described below. For details regarding the primer and adaptor sequences, and an explanation of primer selection criteria, readers should consult the kit documentation.

With AFLP, a restriction digest is performed on a DNA sample (similar to RFLP), and then a set of primer-recognition sequences (adaptors) is used to amplify the restriction fragments using PCR (Zabeau and Vos, 1993). The primers and restriction enzymes used are not specific for a gene or group of genes, but can, theoretically, interact in numerous random places throughout a genome. AFLP is very similar in premise and application to RAPD fingerprinting, which has been used a number of times to compare microbial community structure (Franklin et al., 1999 a & b; Wikström et al., 1999; Xia et al., 1995); the specific use of AFLP for community analysis is discussed in Franklin et al. (2001)

7.2.4.1. Restriction / ligation procedure and preselective amplification

The restriction and ligation steps of the AFLP reaction were performed simultaneously by adding 10 ng of DNA, 2 units of *Mse*I, 4 units of *Eco*RI, and 10 units of T4 DNA ligase (enzymes purchased from New England Biolabs, Beverly, MA) to a reaction mixture containing: 1X T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 25 $\mu g\ ml^{-1}$ bovine serum albumin (NEB, Beverly,

MA)), 0.05 M NaCl, 0.5 μg BSA, 0.2 μM *Eco*RI adaptor, and 2 μM *Mse*I adaptor (PE Applied Biosystems); the total reaction volume was 11 μl. The reactions were incubated for 6 hrs at 37°C, and then diluted by adding 189 μl of $TE_{0.1}$ buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Preselective amplification was performed following the manufacturer's protocol, though the PCR mixture was supplemented with 400 μg ml^{-1} BSA. Successful amplification was verified by agarose gel electrophoresis of 10 μl of PCR product in a 1.5 % agarose gel. The remaining product from the preselctive amplification (10 μl) was then diluted with 190 μl $TE_{0.1}$ buffer.

7.2.4.2. Selective amplification

For the selective amplification, several different combinations of primers were tested; in each case, one *Eco*RI primer, labeled with a fluorescent dye, was paired with one *Mse*I primer. However, the AFLP patterns obtained using the bacterial primer pairs were too complex, and primers from the AFLP Plant Mapping Kit (PE Applied Biosystems) were also tested. These primers were identical to those designed for the bacterial samples, but contained an additional selective nucleotide at the 3' end of the primer. After screening several pairs of primers, two sets were selected for use in this study; the selection was based on the number and intensity of the peaks in the final AFLP fingerprint, as well as the reproducibility of these fingerprints. The primer pairs used were: *Eco*RI-ACA (FAM labeled) with *Mse*I-CAA, and *Eco*RI-AAC (NED labeled) with *Mse*I-CTC.

Selective amplification was performed as directed in the kit documentation, with two modifications. First, the reaction volume was doubled (20 μl total), and, secondly, the PCR reaction mixture was supplemented with 800 μg ml^{-1} BSA. Successful amplification was confirmed by agarose gel electrophoresis (8 μl of PCR product, 1.5% agarose gel). The remaining PCR product was then purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA). To elute DNA from the QIAquick column, 20 μl of elution buffer were added to the center of the membrane, allowed to stand for 1 min, and then centrifuged for 1 min at 13,000 rpm in a tabletop microcentrifuge.

7.2.4.3. Electrophoresis and data collection

After purification, the selective amplification products were resolved using an ABI Prism 310 Genetic Analyzer. For the FAM-labeled products, 10 μl of PCR product were mixed with 1 μl of size standard (GeneScan 500 ROX, PE Applied Biosystems) and 14 μl of deionized formamide. For the NED-labeled products, 1.5 μl of PCR product were mixed with 1 μl of the size standard and 22.5 μl of deionized formamide. These mixtures were denatured by heating to 95°C for 5 min, and then quick-chilled on ice. The samples were analyzed with the following electrophoresis parameters: 10-sec injection time, 15-kV injection voltage, 13-kV run voltage, and 30 min run time.

The electropherograms of the AFLP products were analyzed using the Genotyper software (PE Applied Biosystems), and the presence or absence of each peak in each sample was coded as 1 or 0. The data from the two primer pairs were pooled into a single

large dataset for all further analyses. Collectively, these primers produced a total of 331 bands, and an individual sample contained between 20 and 210 bands. The average number of bands observed for an individual sample was 88.

The Jaccard coefficient was used to calculate the relative similarity between each set of samples, based on the proportion of positive bands shared by a sample pair (Sneath and Sokal, 1973). The similarity matrix was then converted into a dissimilarity matrix by subtracting each value from 1. The dissimilarity matrix represents the relative difference in microbial community genetic structure between each pair of soil samples.

7.2.5. Geostatistical analyses

In most geostatistical analyses, a variance term (usually semi-variance) is calculated between each pair of samples and graphed versus spatial separation to produce a variogram. When the overall spatial structure of multivariate dataset is of interest, researchers may generate plots using a 'resemblance coefficient' for the Y-axis (e.g., a similarity or dissimilarity matrix (Franklin et al., 2002; Mackas, 1984; Underwood and Chapman, 1998)) or information derived from a principal components analysis (Kuzyakova et al., 2001; Saetre, 1999; Saetre and Bååth, 2000; Sokal et al., 1980), rather than a conventional variance estimate. Since the AFLP analyses generated multivariate binary data, it was not possible to calculate semi-variance between sample pairs; instead, pseudo-variograms were created using the "relative dissimilarity" values calculated from the Jaccard similarity matrix. These pseudo-variograms were constructed and analyzed using the same techniques as traditional variograms.

7.2.5.1. Analytical approach

An analytical approach was developed to explore two distinct aspects of spatial variability in these soil microbial communities. First, the overall spatial autocorrelation structure was analyzed in order to quantify the relationship between community variability and spatial separation (lag distance). Data from all sampling locations were included to provide an average portrait of the spatial relationships in the plot. This system was analyzed multiple times, changing the size of the observational window, to study this relationship at different spatial scales. The second portion of the analysis was directed toward trying to understand any changes in spatial pattern and community organization associated with different locations in the field.

For the first set of analyses, data from all of the sampling locations were analyzed to obtain a portrait of the average spatial relationships in this plot. Subsets of this data, varying in maximum separation distance, were also analyzed to quantify autocorrelation at different spatial scales. These scales were named based on relative size, and the following designations were used: plot scale (all sampling locations), large scale (separation distances up to 5 m), small scale (up to 1 m), and fine scale (up to 0.4 m). For each of these different sample groupings, geostatistical analyses of the overall difference in community structure were performed.

For the second set of analyses, local spatial autocorrelation was quantified by analyzing samples from different sections of the plot. These results were used to help understand whether the spatial autocorrelation structure was different in different areas of the field. An analysis of each scale was performed at each of the five different nodes (A

– X (Figure 7.1)): large scale (all samples located within a 2.5 m radius surround each node (maximum separation distance of 5 m)), small scale (all samples located within a 0.5 m radius), and fine scale (all samples located within a 0.2 m radius).

Lastly, in order to determine whether the pattern of spatial variability changed with direction in the field (anisotropy), the data were also grouped into two additional categories. The "North-south" (N/S) analysis included all of the samples collected along the axis between nodes B and D, and the "East-west" (E/W) analysis included all of those points along the line between nodes A and C.

7.2.5.2. Guidelines used in variogram construction

Prior to constructing each variogram, it was first necessary to segregate the data into distance classes by calculating the appropriate number of bins and the appropriate bin width (lag distance). The purpose of "binning" the data was to obtain the maximum resolution (the most detail) at small distances, without being misled by structural artifacts resulting from whatever particular size class was chosen. This approach allowed us to quantify the dominant spatial pattern at each scale, but obscured the autocorrelation structure at smaller distances. For each analysis, lag distance was calculated by considering the maximum separation distance between sample pairs, as discussed in Franklin et al. (2002). Several variograms were then produced and modeled, for a range of different lag distances surrounding this initial estimate, and the results with the best fit (highest R^2), the most reasonable parameter estimates, and the maximum detail are presented here.

One problem with using equal distance classes to construct a variogram is that the number of points in some bins may be quite small (especially bins at the far right of the variogram), so the average associated with one of these classes may not be a valid estimate of the mean at that distance. To help avoid this problem, all bins that contained fewer than 1 % of the total number of points (pairwise comparisons) in each analysis were removed from the experimental variograms prior to statistical modeling.

7.2.5.3. Modeling the experimental variograms

In general, variograms may take one of three different forms: nugget (sometimes called "nugget effect"), linear, and linear-sill. A variogram that is categorized with a *nugget* model is flat, indicating a lack of spatial structure in the data at the scales measured. A variogram that displays a *linear* pattern represents a system where samples are autocorrelated at all of the separation distances measured, and may be modeled with a linear equation: $y = C_0 + bx$, where y is the variance term (in this case, dissimilarity in genetic community structure), x is the spatial separation distance, C_0 is the y-intercept, and b is the slope of the variogram model. Most of the time, these variograms do not pass though the origin of the graph, but display some variability even at separation distances of zero; this value, C_0, is also referred to as "nugget" because it represents the variability in the data that cannot be modeled using the spatial autocorrelation function. This phenomenon may occur because of random sampling variance, experimental error, or variability at other spatial scales.

A *linear-sill* model is a general term used to describe variograms that display increasing variance with increasing separation (conceptually similar to the linear model), and then level off at a *sill*. In our study, all variograms displaying this pattern were modeling using the exponential equation:

$$y = C_0 + C_1[1 - \exp(-3\frac{x}{a})]$$

where y is the variance term, and x is the spatial separation distance. C_0 is a parameter quantifying the nugget effect (the amount of variability at distance = 0), C_1 is a spatially structured component of the model, and a is the range (the distance beyond which variance is no longer a function of spatial separation). The range is sometimes referred to as the correlation length scale (CLS). In the exponential model, the (semi)-variogram approaches its maximum asymptotically, and the range is therefore defined as the distance where the (semi)-variance equals 95% of the sill. The sill (C) is the y value at which the variogram levels off, and can be calculated as: $C = C_0 + C_1$. The ratio of the spatially structured component of the model to the total variability captured by the model (C_1/C) represents the proportion of variability in the dataset that was modeled by the autocorrelation structure function, and is commonly referred to as *spatial dependence*. This value approaches 1 in a strongly spatially structured system, and 0 when no spatial structure is detected with the sampling scale used.

Each of the experimental variograms was modeled using either a linear or an exponential equation. All regressions were performed in SigmaPlot (Version 5.0) and R^2 was used to measure the fit of the model to the data; p values less than 0.05 were considered statistically significant. From the model, C_0, C_1, and a were estimated, and

the sill (C) and spatial dependence (C_1/C) were calculated. Situations in which a linear or exponential model could not be successfully applied ($p > 0.05$) were categorized as "nugget."

7.2.5.4. Kriging

Kriging is a family of generalized least-squares regression algorithms that may be used to estimate the values of a given parameter at unsampled locations, by considering the spatial autocorrelation structure of the variable as determined for the sampled locations. In the variogram analyses discussed above, we used the similarity matrix to describe the overall relationship between samples, and determined the autocorrelation associated with variation in the composition of the entire community. However, it was not possible for us to generate maps using the similarity matrix (we cannot plot paired values across all locations) or to use the original data matrix (1's and 0's) in the kriging. Instead, a principal components analysis (PCA) was performed on the original data in order to generate numerical values describing community structure at each sampling location. The first three principal components (PCs) were used in the kriging, and each describes a portion of the variability in a microbial community structure.

A separate geostatistical semi-variogram analysis was performed on each of the first three principal components to quantitatively describe the spatial autocorrelation structure for each PC. This information was used in the kriging to generate maps of the PC scores with the SADA statistical package (Spatial Analysis and Decision Assistance, Version 3.0.80, University of Tennessee). This approach was also used to generate maps of microbial community structure along each of the main axes of the sampling grid (N/S

and E/W, as analyzed in the directional analysis). In this case, maps were only produced for the first principal component.

7.3. Results

7.3.1. Quantifying multiple scales of spatial autocorrelation within the plot

In the first set of analyses, data from all of the sampling locations were considered in order to obtained an average portrait of the spatial relationships in the plot (Figure 7.2). For the plot-scale analysis, a bin size of 0.5 m was used, and the number of points included in each bin varied from a minimum of approximately 100, for very large separation distances (> 10.5 m), to more than 3000 points for intermediate separation distances. On average, each point on the variogram is the mean of approximately 800 pairwise comparisons. The points on the variogram that were averages of a small number of comparisons (< 1 %) were excluded from the graph and the geostatistical modeling. A similar approach was used for the large (Figure 7.2 B), small (Figure 7.2 C), and fine scale analyses (Figure 7.2 D), where 8288, 2860, and 1670 pairwise comparisons were used, respectively.

Significant spatial autocorrelation was detected at each analytical scale (Table 7.1), and could be modeled using either the exponential (plot and large scale) or linear equations (small and fine scale). The plot-scale analysis showed that the overall spatial pattern was organized with a range of 6.3 m at this site, and the large-scale analysis showed another level of organization with a range of 2.0 m. The small and fine-scale analyses displayed spatial autocorrelation, but range estimates could not be made because a sill was not reached within either analytical extent.

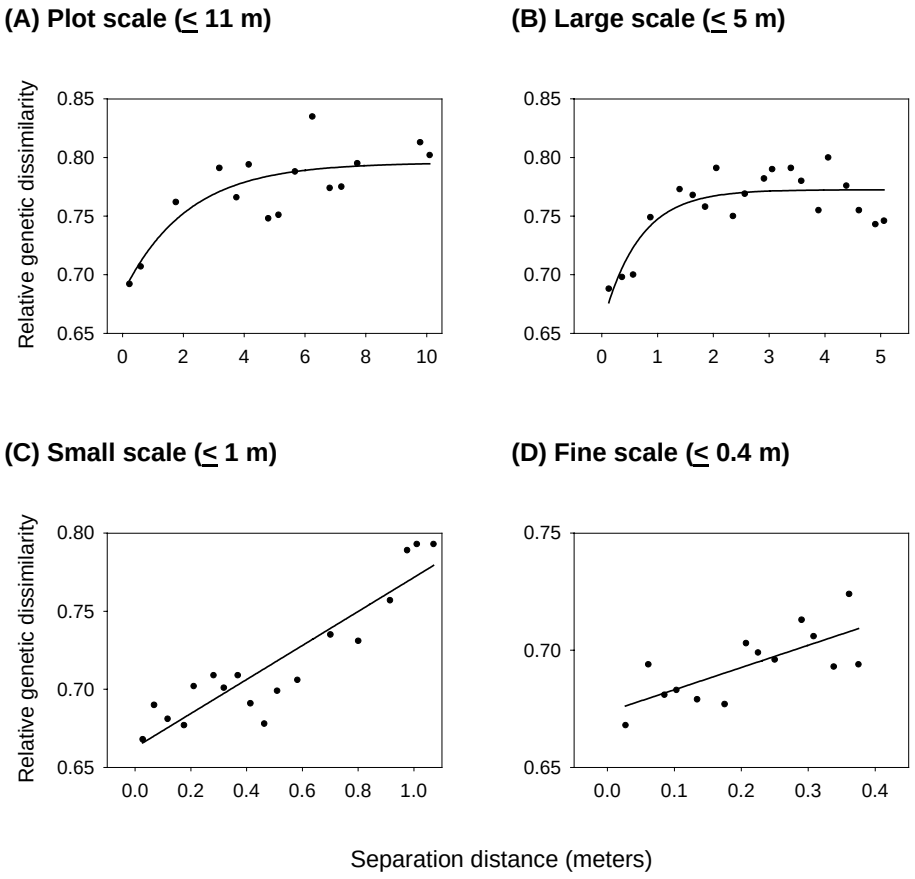

Figure 7.2. Variograms used to model the overall spatial autocorrelation structure at each analytical scale. Data from all of the sampling locations were included to obtain an average portrait of the spatial relationships in the plot. (A) Plot scale, all sampling locations. (B) Large scale, separation distances less than 5 m. (C) Small scale, separation distances less than 1 m. (D) Fine scale, separation distances less than 0.4 m.

Table 7.1. Summary of results from geostatistical analyses of community structural similarity (AFLP profiles).

Scale and extent	Node	Model type	R^2	p	Nugget	Sill	Spatial dependence	Range (m)
Plot (0.025 – 11 m)	Entire plot	Exponential	0.67	0.001	0.68	0.80	0.14	6.3
Large (0.025 – 5 m)	Entire plot	Exponential	0.70	< 0.0001	0.66	0.78	0.15	2.0
	A	Nugget			0.72			
	B	Exponential	0.74	0.002	0.67	0.80	0.16	1.3
	C	Exponential	0.93	< 0.0001	0.63	0.76	0.17	3.3
	D	Exponential	0.93	< 0.0001	0.65	0.83	0.21	1.9
	X	Exponential	0.96	0.002	0.56	0.82	0.32	1.8
Small (0.025 – 1 m)	Entire plot	Linear	0.83	< 0.0001	0.66			> 1.0
	A	Nugget			0.73			
	B	Exponential	0.92	0.006	0.66	0.76	0.12	0.6
	C	Exponential	0.63	0.03	0.60	0.70	0.15	0.6
	D	Nugget			0.73			
	X	Nugget			0.65			
Fine (0.025 – 0.4 m)	Entire plot	Linear	0.52	0.0034	0.67			> 0.4
	A	Nugget			0.73			
	B	Exponential	0.50	0.03	0.66	0.72	0.09	0.3
	C	Linear	0.76	0.01	0.61			> 0.4
	D	Linear	0.65	0.05	0.72			> 0.4
	X	Linear	0.45	0.009	0.6			> 0.4

Because of the techniques and conventions used to construct these variograms, the smaller-scale autocorrelation structure of these communities was usually not visible in the variograms constructed for larger spatial extents. For example, in the plot-scale analysis, the data were binned with a lag distance of 0.5 m; this action made it impossible to detect the autocorrelation structure at the fine (≤ 0.4 m) and small scales (≤ 1 m). Similarly, the resolution associated with this bin size was not sufficient to allow us to accurately model spatial autocorrelation at the large scale (≤ 5 m). In order to study the autocorrelation structure at these other spatial scales, only the relevant sections of the variogram were considered. It is generally acceptable to analyze subsets of a variogram in this way, so long as there are enough data. A geostatistical "rule of thumb" suggests that each distance class should contain at least 30 pairs of points; however, the greater the number of points, the greater the statistical reliability (Rossi et al., 1992). This guideline was far exceeded in all analyses, except the fine-scale analysis for the individual nodes.

7.3.2. Comparing spatial autocorrelation in different regions of the field

When the data from the different regions of the plot were analyzed separately, using each node (A – X) as a center point, other CLS were detected (Table 7.1). For each scale and each sampling location, variograms were constructed using either 741, 528, or 210 pairwise comparisons (per node) for the large, small, and fine-scale analyses, respectively. In general, each dataset displayed an obvious linear or linear-sill pattern, and the appropriate model was applied. However, in a few cases, when visual interpretation of the data was unclear, it was necessary to fit the variogram with both a

linear and an exponential equation, and use statistical criteria (R^2 and p value) to determine the most appropriate model.

For any given scale of analysis, the results for the different nodes were usually similar (Table 7.1). At the large scale, spatial autocorrelation was modeled at 4 of the 5 nodes, using an exponential equation; range estimates varied between 1.3 and 3.3 m, and the average of the different estimates was 2.0 m. For the small scale, significant models of spatial autocorrelation were only determined at two of the nodes, which produced identical range estimates of 0.6 m. At the fine scale, the exponential model was applied to node B, and the range estimate was 0.3 m. At nodes C, D, and X, a linear model was appropriate, and indicated that the communities are spatially autocorrelated with a range greater that 0.4 m, which was the maximum separation distance used at that level of analysis.

In general, the results for the different sampling locations (nodes) were similar, though each node displayed a unique multi-scale pattern of organization (Table 7.1). The same patterns of spatial organization were found at nodes D and X (identical CLS for each scale), and the patterns observed at nodes B and C were very similar. At node A, spatial autocorrelation was not detected for any of the analytical scales.

7.3.3. Directional variograms

In order to determine whether the pattern of spatial variability changed with direction in the field, the data were also analyzed along each axis of the sampling grid. For the E/W transect, the variogram could be modeled using a linear equation (Table 7.2, Figure 7.3 B). For the N/S transect, multiple scales of spatial autocorrelation were

Table 7.2. Summary of results of the geostatistical analyses of the directional variograms.

Direction	Portion modeled	Model type	R^2	P	Nugget	Sill	Spatial dependence	Range (m)
North-south[a]								
Section 1	0 – 4 meters	Exponential	0.92	< 0.0001	0.65	0.84	0.23	1.8
Section 2	5 – 11 meters	Exponential	0.64	0.01	0.68	0.84	0.18	1.8
East-west [b]								
Entire length	0 – 11 meters	Linear	0.72	< 0.0001	0.69	-	-	> 11.0

[a] North/south refers to all points along the line from node B to node D.
[b] East/west west refers to all points along the line from node A to node C.

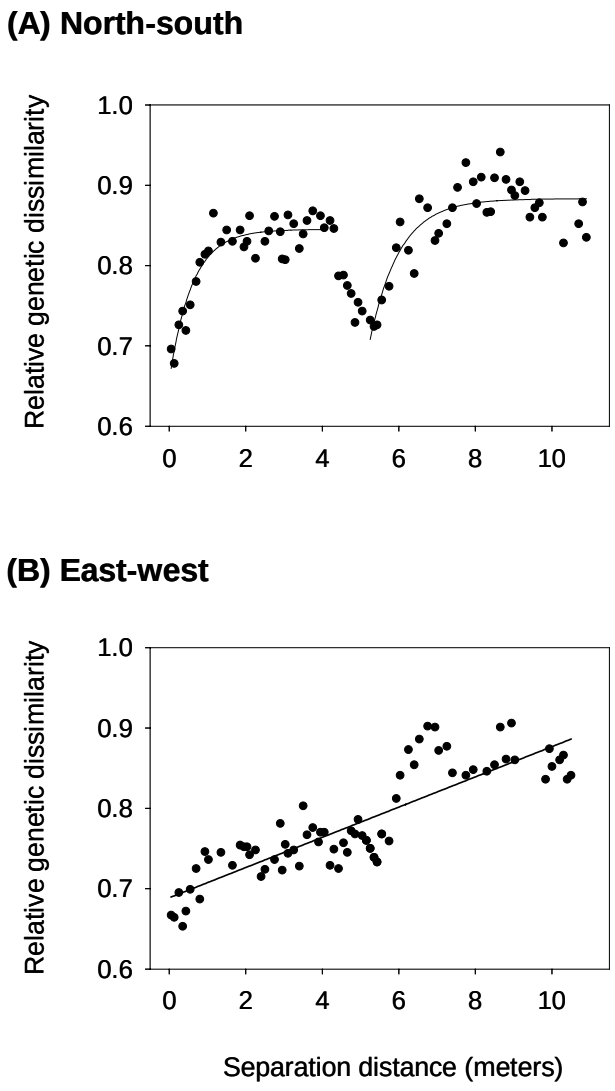

Figure 7.3. "Directional variograms" used to model the overall spatial autocorrelation structure along each axis of the sampling grid.

observed within a single variogram (Figure 7.3 A); the variogram was divided into two regions, which were analyzed separately to estimate the range and spatial dependence using an exponential model (Table 7.2). A repeating pattern was evident, and each of the patches had a CLS of 1.8 m beyond the minimum (nugget) value. The nugget (section 1: 0.65, section 2: 0.68) and sill values (both are 0.84) for the two patches were identical, suggesting that the same amount of total variability was associated with each patch.

7.3.4. Kriging

Kriging was used to generate maps of the spatial distribution of microbial community structure for the entire plot (Figure 7.4), and for each axis in the directional analyses (Figure 7.5). First, the AFLP profiles were analyzed using PCA, and the sample scores from the first three principal components were used as derived variables in the geostatistical modeling (results not shown). Three separate maps were generated at the plot scale, one based on each PC. Together, these components explained 26.4 % of the variance in microbial community structure (PC1: 12.6 %, PC2: 8.3 %, PC3: 5.5 %). For each directional analysis, an additional PCA was performed using data from only the respective sampling locations. The first PC from each analysis was then used to generate a map of microbial community structure along each axis using ordinary kriging. The first PC explained 18% of the variance among the samples located along the N/S axis, and 15% of the variance among the samples located along the E/W axis.

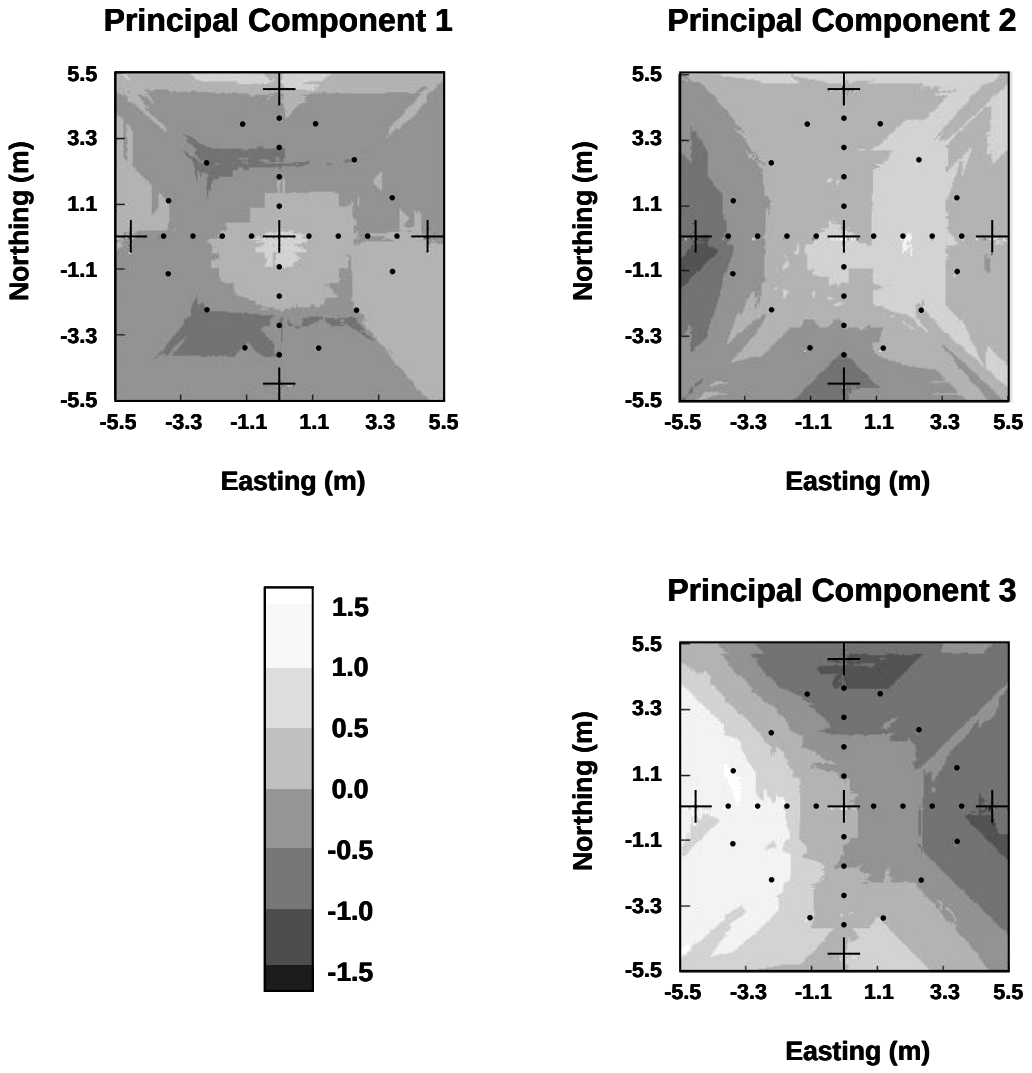

Figure 7.4. Kriged maps showing the distribution of microbial community structure, as described by the first three principal components, across the plot. Each graph is oriented to match Figure 7.1A; sampling nodes are indicated with '+', and perimeter and diagonal sampling locations are marked with '•'.

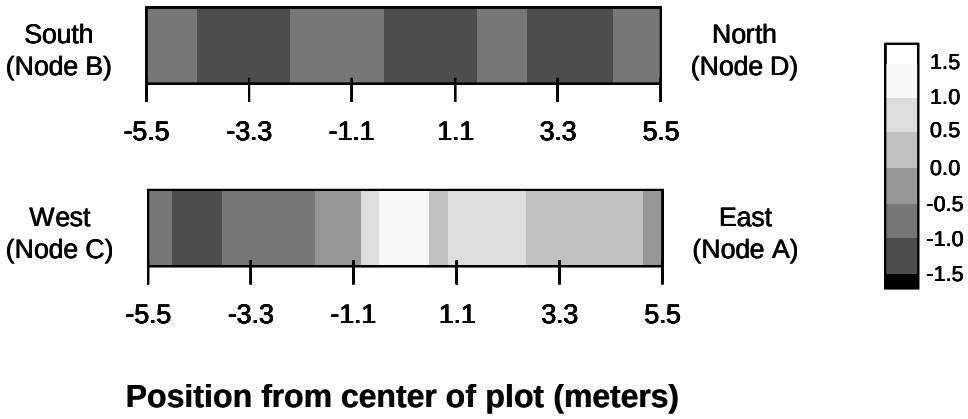

Figure 7.5. Kriged maps showing the distribution of microbial community structure, as described by the first principal component, for each directional analysis.

7.4. Discussion

In order to more fully characterize the spatial variability of microbial systems, studies that make use of several different scales of measurement are necessary. In this research, multi-scale analysis of the spatial distribution of a soil microbial community revealed several different scales of organization, ranging from 30 cm to more than 6 m. In some locations, it was possible to identify and quantify up to four different CLS. The patch size estimates varied some at the different sampling locations across the plot (different nodes) indicating that the patterns of spatial organization at a particular level (spatial scale) are not necessarily fixed across this system.

When the multi-scale approach was applied to analyze the entire plot, two distinct scales of organization were detected (large scale: 2.0 m, plot scale: 6.3 m). Multiple scales of spatial organization were also visible on the kriged maps, and each map showed a different spatial pattern (Figure 7.4). The PCA used in the construction of these maps reduced the complex AFLP fingerprints into a set of derived variables, each of which explains a portion of the pattern present in the AFLP data. In this way, each PC describes a different aspect of the variability among the microbial communities, and the kriging results indicate that those distinct aspects have different patterns of spatial organization. These distinct patterns may develop as separate populations or groups of organisms respond to the spatial distribution of different environmental variables.

The map generated for the first PC shows a patchy structure organized around the center of the plot (node X). The patch in the center of the plot has a diameter of approximately 1.5 – 2 m, the CLS detected in the large-scale variogram analysis, and the next surrounding ring has a diameter of 5 – 6 meters, which corresponds well to the CLS

detected at the plot-scale. One possible explanation for this bull's-eye pattern is that some aspect of the environment at the center of the plot is unique (e.g., a different vegetation patch, a hill or mound, or a large application of fertilizer), and the map shows the variation in community structure along a gradient away from this aberration. In contrast, the maps generated from the second and third PCs reveal a very different spatial pattern. The portion of the communities represented on these maps may be responding to a suite of variables that are spatially structured as a gradient extending from the NE corner to the SW corner of the plot.

In addition to quantifying the overall pattern of spatial organization in this system, our study was designed to evaluate how variable the autocorrelation structure was in different locations. In general, the results for the different sampling locations (nodes) were similar and multiple CLS were detected, though spatial autocorrelation was not detected at node A. The samples collected at this location contained an unusually large amount of plant and root material, which should have been removed by sieving, but could have contaminated the DNA extracts. However, given that the AFLP profiles for node A were not significantly different from those obtained at other locations, we feel this is unlikely. Instead, these results may be correlated with some environmental heterogeneity that altered the spatial organization, but not the overall composition, of the communities in this region of the field.

Spatial dependence is the percent of total model variance that is explained by the spatial autocorrelation function. When this value is low (all variance is nugget), it indicates that most of the spatial dependence occurs at distances greater or smaller than

the scale of study, or that the measurement error associated with the analysis is high (Isaaks and Srivastava, 1989). In the present study, the spatial dependence for any single location (node) or particular analytical scale ranged from 0.09 to 0.32. These values are consistent with other studies considering community structure (Cavigelli et al., 1995; Franklin et al., 2002). For example, when Saetre and Bååth (2000) performed a geostatistical analysis of the overall microbial community structure in a forest soil (using PLFA), the spatial dependence varied between 0.12 and 0.25 for an analytical scale of 0.2 to 20 m. The values discussed above refer to the spatial dependence for the analysis of a single spatial scale. However, if the spatial dependence in the present study is summed across each of the four analytical scales (either for each node, or for the entire plot), the total estimate increases to between 0.35 and 0.45.

Communities at this site are expected to display additional spatial structure at scales larger than the maximum separation distance used (11 m) and at distances smaller than the minimum sampling interval (2.5 cm). In this study, it would have been useful to analyze multiple subsamples from each field sample to determine the variability within a sampling unit. This would have provided additional information regarding community variability at small spatial scales, and would have functionally represented a separation distance of zero. Unfortunately, increasing the number of analyses was not feasible for this study. Recently, Ellingsøe and Johnsen (2002) considered the influence of soil sample size on the analysis of bacterial community structure (using DGGE), and found that sample size did influence their assessment of community structure for smaller sample sizes (0.01 and 0.1 g of soil), but less so for larger sample sizes (1.0 and 10.0 g). In our

study, each DNA extraction was performed on a single 0.25 g subsample, which may not have been sufficient to completely categorize the community variability of each field sample (5 – 10 g).

In the directional analyses, the variograms (Figure 7.3) matched up very well with the kriged maps generated from the PC scores (Figure 7.5). In the E/W variogram, a linear pattern was observed; communities that were near-by along this axis were more similar to one another than they were to communities at greater separation distances, though all samples along the transect were spatially autocorrelated. On the map, patches/communities that are nearby have similar PC scores (more similar grayscale values), but there is very little repetition of an individual community "type." There is a general gradient along the axis that corresponds to the gradient observed in the maps for the entire plot (Figure 7.4), and it is likely that the same environmental factors are responsible for this pattern in both situations.

Along the N/S axis, two types of communities dominate on the map of community structure (Figure 7.5). The patch size for these communities is about 2 m, which is the CLS calculated from the nested variogram (Figure 7.3). It is important to note that, though the kriged map shows only two dominate communities, with PC scores ranging from –1.5 to – 0.5, a frequency histogram of the entire set of PC scores showed a normal distribution with values ranging from – 1.5 to 2.5 (results not presented). The presence of a regular and repeating spatial pattern along this axis suggests that the microbial communities may be partially structured in response to some agricultural or land management activity that occurs in at fixed intervals in the field. For example, it has been suggested that spatial structure may exist in agricultural soils in association with

crop rows and aisles (Ettema and Wardle, 2002; Robertson et al., 1997; Stoyan et al., 2000), and compaction due to wheel traffic may impact microbial activity (Parkin, 1993); however, the CLS of 2 m is likely too large to correspond with these particular features.

Often, when scientists research and discuss the existence of multiple scales of spatial organization in microbial systems, they are referring to the presence of patterns over a very wide range. For example, Parkin (1993) discussed four main scales of interest: microscale, plot scale, field or landscape scale, and regional scale, and Ettema and Wardle (2002) primarily focused their recent review on the distribution of soil properties and biota in distance classes of tens to hundreds of meters, cm to meters, and at microscopic scales. The research presented here demonstrates that a single variable can manifest an incredible amount of spatial structure, at multiple scales, *within* these broad classifications. For example, at node B, four different CLS were detected: 30 cm, 60 cm, 1.3 m, and 6.3 m. This is a remarkable degree of spatial variability for a pedological homogeneous site that has been plowed and cropped as a single field for several years. Variability such as this is likely to exist in most ecosystems, and should be considered when making inferences about ecological relationships and when developing sampling strategies for the environment (Robertson et al., 1997).

While many ecological theories and models acknowledge that elements that are close to one another in space or time are more likely to be influenced by the same generating processes, the classical statistical procedures employed to analyze these phenomena assume an independence of observations. Violations of the assumption of independence and inappropriate application of these statistical procedures to spatially autocorrelated data may lead to incorrect conclusions (Dutilleul, 1993; Legendre and

Legendre, 1998; Murray, 2001). For example, Franklin et al. (2002) found that estimates of microbial abundance obtained using spatially autocorrelated data were significantly different from those obtained using independent samples. The varying degrees of autocorrelation shown in the present study emphasize that sampling approaches and experimental designs may need to consider the impact of spatial autocorrelation, depending on the ecological question of interest. Because it is not always feasible to first do an extensive reconnaissance survey, and because the results presented here suggest that one is not likely to avoid the impact of spatial autocorrelation, a possible solution is to include spatial separation as a part of routine data collection. An initial analysis of this information can then be used to determine the influence of spatial autocorrelation on the dataset. If significant spatial structure is found, this information must be considered as a variable and incorporated into the subsequent data analysis; if not, traditional parametric statistical techniques may be appropriate.

Most of the previous work examining spatial variability in agricultural systems has been performed by soil scientists who were interested in understanding the distribution of physical and chemical properties in order to assess soil quality or determine the impact of land management practices (Castrignanò et al., 2000; Dobermann et al., 1995; Robertson et al., 1997; Savin et al., 2001; Stenger et al., 2002). These studies have shown that spatial autocorrelation is a common feature of such systems. The spatial variability associated with microbial communities is less frequently studied (Robertson et al., 1988; Robertson et al., 1997; Savin et al., 2001), and, in general, efforts to link agricultural soil properties and microbiological properties have been unsuccessful. To our knowledge, the work presented here is one of the first studies

of spatial organization of community structure in this type of environment, and is unique in its consideration of multiple scales of autocorrelation. The results indicate that microbial communities may have several nested levels of organization, even within the cm to meter scale analysis. Different subsets of the community were distributed differently across the plot, and this is thought to be due to the variable response of individual populations to the spatial heterogeneity associated with different soil properties (or groups of properties). Future studies that focus on comparing the spatial structure of microbial communities with that of environmental properties may yield new insights into how communities develop in soil systems, and what factors may be important in maintaining and regulating soil ecosystem function.

Chapter 8.

The role of spatial variability in analysis of microbial community patterns: using multi-scale analysis to compare community structure and soil environmental properties in an agricultural field

Abstract

In an effort to better understand the factors that influence microbial community organization at various spatial scales in soils, the distribution patterns associated with several microbial community and soil physical-chemical properties were compared for an agricultural wheat field. Soil samples were collected with separation distances ranging from 2.5 cm to 11 m, and the multi-scale spatial distributions of soil carbon (C), nitrogen (N), organic matter (OM), texture (sand, silt, and clay content), and bacterial abundance were studied and compared with previously published analyses of microbial community structure (Franklin and Mills, 2003, FEMS Microbiol. Ecol. 44:335-346). Geostatistics demonstrated the presence of spatial autocorrelation for all of the microbial and environmental variables, and kriging was used to generate maps of the spatial distribution of each parameter. The maps of soil texture displayed a large-scale gradient pattern across the sampling plot, while those for C and N showed a more patchy structure, with multiple levels of autocorrelation, nested within this larger-scale gradient. These patterns were quite similar to those previously observed for community structure. Simple causal modeling using Mantel and partial Mantel tests was used to study the direct relationship between each environmental property and each microbial community property, and the results indicate that there is a strong relationship between these two sets of variables, in excess of their shared spatial correlation. In general, soil C and N content was strongly correlated with community structure at all of the scales considered, while other properties (OM and texture) were only correlated with specific subsets of the community. The models developed for larger spatial scales were more complex and indicated a stronger role of spatial heterogeneity in controlling microbial community structure, compared to

models developed for finer spatial scales (≤ 40 cm). These results demonstrate the value of considering multiple spatial scales when studying environment-community interactions, and this approach was particularly useful for determining what physical and chemical soil properties helped control the development of this microbial community. Such information will allow scientists to better predict a community's responses to external factors and the effect of its activities the environment.

8.1. Introduction

The factors affecting the size and composition of microbial communities in soils are poorly understood. This is partially due to methodological constraints that limit our ability to study microbial diversity, and further complicated by the fact that any investigation of soil is faced with the problem that the substrate is highly variable on small scales, both horizontally and vertically. This variability may result from a complex set of geological, pedological, and biological processes, as well as different types of land use. Within a single field, the distribution of soil physical and chemical properties is the result of many superimposed processes acting at different spatial and temporal scales. These properties may cause differences in both the structure and function of soil microbial communities, and it is thought that the relative role of different forces may vary across scales.

The assessment of spatial and temporal changes in microbial community structure, induced by biotic or abiotic factors, is a major research objective of environmental microbiology. Obtaining information regarding community composition across various scales is an important first step in understanding microbial community

structure-function relationships, and knowledge of the spatial patchiness of bacteria is important for determining the appropriate sampling scales and for addressing basic ecological questions. However, despite the assumed importance of spatial variability in soil microbiology, studies that specifically consider spatial scale when examining the distribution patterns of microorganisms, and the possible causes of these patterns, are rare. Moreover, most of the studies that have investigated the spatial variability of soil physical, chemical, and microbiological properties only focus on a single spatial scale (Morris, 1999; Mottonen et al., 1999; Robertson et al., 1988; Saetre, 1999; Savin et al., 2001), even though spatial heterogeneity in community composition, and the analysis of its relationship to habitat heterogeneity, is scale-dependent (Pinel-Alloul et al., 1995). Because of the hierarchical nature of spatial variability, any investigation of the distribution of populations and assemblages along environmental gradients should be integrated with a multi-scale analysis of spatial variability in order to more fully represent the complexity of natural systems (Benedetti-Cecchi, 2001).

Recently, scientists have begun to focus on multi-scale comparisons when analyzing community-environment relationships, and have found evidence for nested scales of spatial structure for both soil physical-chemical properties and the associated microbial communities (Ettema and Wardle, 2002; Morris and Boerner, 1998 & 1999; Nunan et al., 2002; Robertson and Gross, 1994; Robertson et al., 1997; Saetre and Bååth, 2000; Stenger et al., 2002). In a recently published work, we explored the multi-scale spatial distribution of microbial community structure in an agricultural wheat field, and demonstrated that several scales of spatial autocorrelation may exist within the cm- to 10-m scale (Franklin and Mills, 2003). Nested levels of spatial autocorrelation were

observed (ranging from 30 cm to more than 6 m), and, in some locations, up to four distinct ranges of spatial influence were observed. The presence of nested scales of variation suggests that the environmental factors regulating the development of the microbial communities in this soil may operate at different scales, and a simultaneous analysis of the multi-scale spatial variability of microbial community structure and soil microenvironment could help identify these factors and determine their relative importance. The purpose of this manuscript is to present results concerning the multi-scale spatial distribution of soil environmental properties, and to compare them to the previously reported data describing microbial community structure.

Using geostatistical analyses and kriging, the spatial distribution of several physical-chemical soil properties was studied (total soil carbon and nitrogen, organic matter content, and soil texture). Spatial structure was present at multiple scales in this system, and different environmental variables displayed different spatial patterns; some of these patterns were very similar to those previously observed for microbial community structure. Simple causal modeling was used to study the direct relationship between each environmental variable and each microbial community property to determined whether the similarity in these patterns was significant, or spurious and the result of a shared spatial correlation. The results indicted that there is a strong relationship among microbial community structure and the soil properties, and that the influence of spatial variability on these relationships changes at different spatial scales. Moreover, different subsets of the microbial community responded differently to the various environmental properties.

8.2. Materials and Methods

8.2.1. Site description and sample collection

Soil samples were collected from an agricultural field on the eastern shore of Virginia (USA). The field was located along the lagoonal shoreline of the Delmarva Peninsula, approximately 700 m from the edge of the open water (37° 17.62′N, 75º 55.53′W). The soil at this site was very sandy, with low organic matter content, and had been plowed as a single crop for many years. At the time of sampling (May 2000), the field was planted with durum wheat (*Triticum turgidum*), which was approximately 75 days old.

Samples were collected with separation distances ranging from 2.5 cm to 11 m, following the sampling scheme detailed below. At each sampling location, the loose layer of surface material was removed, and a small hole (1.5 cm diameter) was dug to collect 5 – 10 grams of soil. The samples were placed on ice for transport to the lab, where they were sifted (approximately 750-μm mesh size) to remove gravel, plant, and root material, and stored at -80°C.

8.2.2. Sampling scheme

The basic sampling design was a square with 7.1-m edges and 10-m diagonals (Figure 8.1). Samples were collected at regular intervals around the perimeter of the block (1.8-m separation distance), and at 1-m intervals along the diagonals. At each node (A, B, C, D, and X), more concentrated sampling efforts were employed.

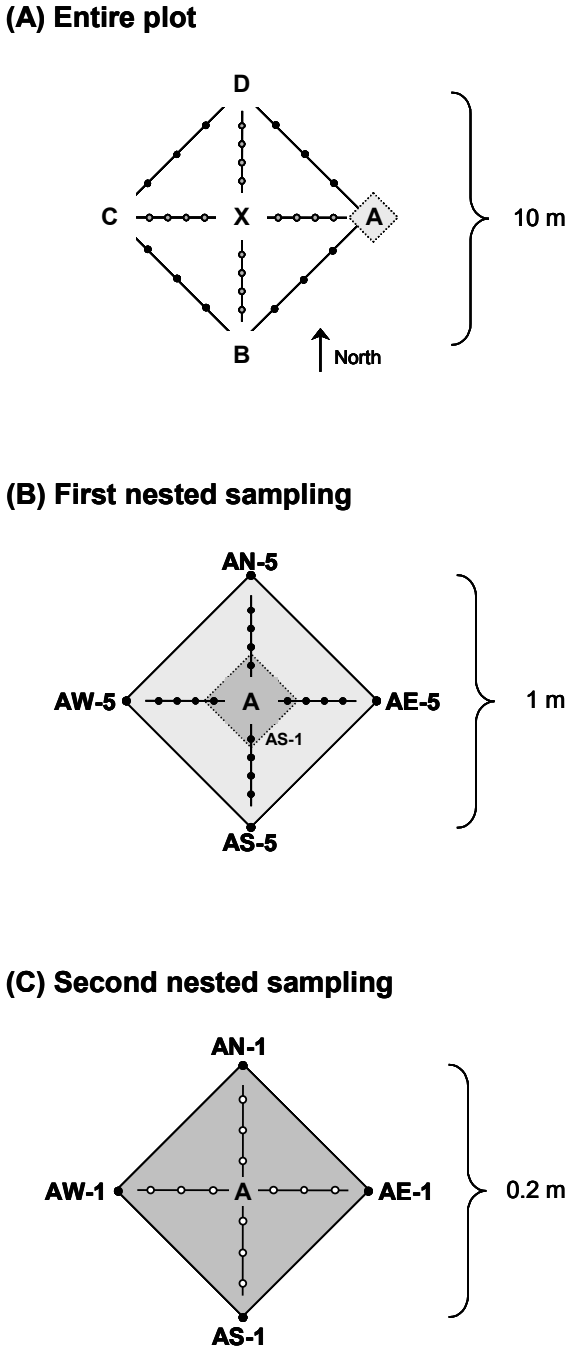

Figure 8.1. Map of the sampling scheme. (A) The sampling area was a 50 m^2 square (diamond) with 10 m diagonals. Around the perimeter of the square, samples were collected at 1.8 m increments, and at 1 m increments along the diagonals. At each node (A – X), more concentrated sampling efforts were employed. A nested sampling pattern was applied at each location, and node A is presented in the figure as an example. Additional samples were collected at 10 cm increments (B) and 2.5 cm increments (C) in a cross shape surrounding each node

Nested within the original sampling grid, a second set of samples were collected at 10-cm increments in a cross shape surrounding each node. Five samples were collected in each direction (north, south, east, and west) away from the center node. Nested within this area, a third set of samples was collected at 2.5-cm increments around each node, following the same pattern (2.5, 5.0, 7.5, and 10 cm in each direction). A total of 193 soil samples were collected, 33 at each node and 28 at larger separation distances.

8.2.3. Analysis of soil properties

Originally, these samples were collected with the research goal of examining the spatial distribution of the soil microbial communities (Franklin and Mills, 2003). The results of that study prompted us to further analyze (the remains of) the initial samples, though this data collection was limited in two ways. First, the soil samples we collected were necessarily small (5 – 10 g), to permit an analysis of small-scale (cm) spatial variability; this small sample size restricted the number and type of soil physical-chemical properties that could be analyzed. Second, because these analyses were performed on stored, frozen samples, certain parameters could not be evaluated (e.g., moisture content). Within these constraints, we selected environmental properties that were likely to directly influence soil microbial communities, and measured total soil carbon (C) and nitrogen (N) content, organic matter content (OM), and soil texture (sand, silt, and clay fractions).

The C and N content of the soil was determined using an elemental analyzer (CE Elantech, Lakewood, NJ). For each sample collected in the field, an approximately 1-gram subsample was oven dried and ground using a mortar and pestle. From this

material, two subsamples of 30 mg each were analyzed, and the percent carbon and percent nitrogen were determined.

A modified version of the pipette method was used for particle-size determination, based on the technique proposed by Kettler et al. (2001). Percent sand, silt, and clay were calculated, and a small subsample of soil was also analyzed for OM content by determining the amount of material lost upon ignition (450°C, 24 hrs).

8.2.4. Analysis of microbial communities

Several different techniques were used to characterize the microbial communities associated with each sample, including two DNA fingerprinting techniques (Amplified Fragment Length Polymorphism (AFLP) and Terminal Restriction Fragment Length Polymorphism (T-RFLP)), to compare community structure, and Acridine Orange direct counting (AODC), to determine bacterial abundance. AFLP, used to obtain a picture of the overall differences in microbial community structure, was applied to all 193 samples. Those results have been discussed in detail in Franklin and Mills (2003), and will only be included in this manuscript as they relate to the present analysis of other microbial properties and environmental variables. The T-RFLP and AODC techniques were applied to a subset of samples.

8.2.4.1. T-RFLP

Two samples from each node (A, B, C, D, and X) were selected, DNA was extracted and quantified (Franklin and Mills, 2003), and T-RFLP was performed as described in Liu et al. (1997). The eubacterial primers 27 Forward (5' AGA GTT TGA

TCC TGG CTC AG 3') and 1492 Reverse (5' GGT TAC CTT GTT ACG ACT T3') were used, and the 5' end of the 27 Forward primer was fluorescently labeled with 6-FAM (5[6]-carboxy-fluorescein) (Operon Technologies Alameda, CA). The PCR reaction mixture included: 1X PCR buffer, 200 mM of each dNTP, 2.0 mM $MgCl_2$, 1.0 μM of each primer, 0.4 μg μl^{-1} bovine serum albumin (BSA), and 1.25 units of Ampli Taq DNA polymerase (PE Applied Biosystems, Foster City, CA) per 50 μl reaction. The thermal cycling conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of: 94°C for 0.5 min, 58°C for 1 min, 72°C for 2 min, with a final elongation at 72°C for 10 min. Separate portions (10 μl) of the PCR product were then digested with either the *HhaI* and *RsaI* restriction enzymes (New England Biolabs, Beverly, MA).

Data were collected using an ABI Prism 310 Genetic analyzer. The presence or absence of each terminal restriction fragment (T-RF) in each sample was determined and recorded as a matrix of 1's and 0's. Relative similarly was calculated between each sample pair using the Jaccard coefficient (Sneath and Sokal, 1973), and converted to a dissimilarity measure using the following relationship: Dissimilarity = 1 – Similarity.

8.2.4.2. Microscopic counts of total bacterial abundance

After analysis of community structure and soil properties, microscopic counts of total bacterial abundance were carried out on any samples with material remaining (59 samples, collected from locations throughout the plot). Approximately 0.5 g of sample was combined with 50 ml filter sterilized water, and blended for 1 min. The samples

were allowed to settle for 2 min, and a 1 ml aliquot was then removed. AODC was performed (Bottomley, 1994; Hobbie et al., 1977), and the number of cells g^{-1} dry weight of soil was calculated.

8.2.5. Data analysis

The objectives for this study can be classified into two general categories: (i) to test for the presence of spatial autocorrelation in the environmental data and to describe any spatial structure, and (ii) to explore the relationship between microbial community structure, soil microenvironment, and spatial separation. A multi-scale approach was used, and each objective was addressed at each analytical scale. Several different statistical techniques were employed to accomplish these goals including geostatistical semi-variogram analysis, kriging, and causal modeling using Mantel and partial Mantel tests. A brief summary of each of these techniques is presented below, followed by specific information regarding the application of each procedure to our dataset. For a comprehensive introduction to these statistical methods, readers should consult Legendre and Legendre (1998).

Before any spatial statistics were computed, an exploratory data analysis was performed for each of the environmental variables and the bacterial abundance data (Table 8.1). Summary statistics including the mean, range, standard deviation and coefficient of variation were calculated (SPSS Version 10.1.0), and a Kolmogorov-Smirnov test demonstrated that each variable was normally distributed (results not presented).

Table 8.1. Basic statistics for the environmental variables and bacterial abundance.

Parameter (%)	**Min**	**Max**	**Mean**	**SD**	**CV (%)**
Carbon (C)	0.35	0.89	0.60	0.104	17.3
Nitrogen (N)	0.03	0.09	0.06	0.008	12.9
Organic matter (OM)	0.61	1.58	1.05	0.21	20.0
Sand	61.6	78.1	70.1	3.35	4.6
Silt	19.6	34.4	26.1	2.63	10.1
Clay	0.36	5.46	2.69	1.12	41.6
Bacterial abundance *	1.0×10^9	3.1×10^9	1.9×10^9	5.4×10^8	28.4

* Cells gram^{-1} dry weight of soil.

8.2.5.1. Outline of multi-scale approach

An analytical approach was developed to quantify the spatial variability in this system at multiple scales. For the first set of analyses, the relationships among all pairs of sampling locations were considered, to obtain an average portrait of the overall spatial variability in this plot. Subsets of these data, varying in maximum separation distance, were then analyzed to quantify autocorrelation at different spatial scales. These scales were named based on relative size, and the following designations were used: plot scale (all sampling locations (separation distances ≤ 11 m)), large scale (≤ 5 m), small scale (≤ 1 m), and fine scale (≤ 0.4 m). Because of the techniques and conventions used in geostatistical analyses, smaller-scale spatial autocorrelation structure is usually not detected when analyses are performed on larger spatial extents (for more details, see Franklin and Mills (2003)). Therefore, in order to study the autocorrelation structure at these other spatial scales, only the relevant portions of the data were considered.

8.2.5.2. Characterization of spatial structure

8.2.5.2.1. Variogram analysis of environmental data

Geostatistical variogram analysis was used to examine the spatial autocorrelation structure across the plot for each individual environmental variable at each analytical scale. Experimental variograms were constructed using the semi-variance statistic and the procedure outlined in Franklin and Mills (2003). In order to make meaningful

comparisons between data with disparate measurement units and/or different levels of spatial variability, the plots were standardized by dividing each variogram value by the overall sample variance prior to geostatistical modeling.

Each experimental variogram was modeled using either an exponential or linear equation; in some cases, neither model was appropriate and the variogram was classified as "nugget" (no spatial autocorrelation structure at the scale considered). Variograms displaying a linear pattern represent a system where samples are autocorrelated at all of the separation distances measured. The exponential model was used to describe variograms that displayed increasing variance with increasing spatial separation (conceptually similar to a linear model), and then reached a plateau, usually called the "sill", which is roughly equal to the sample variance. R^2 was used to measure the fit of each model to the data using SigmaPlot (Version 5.0), and p values less than 0.05 were considered statistically significant.

From the model output, it is possible to calculate several parameters that are useful for describing spatial autocorrelation structure. This manuscript will focus on the interpretation of three parameters: the range, spatial dependence, and the nugget effect. The *range*, sometimes referred to as "patch size" or "correlation length scale" (CLS), is the separation distance beyond which variance is no longer a function of spatial separation. It is the "range" over which spatial autocorrelation can be detected, and samples at larger separation distances are not considered to be autocorrelated (for a given analytical scale). *Spatial dependence* is the ratio of the spatially structured component of the model to the total variability captured by the model. It represents the proportion of variability in the dataset that can be accounted for by the spatial autocorrelation structure

function. This value approaches one in a strongly spatially structured system, and zero when no spatial structure is detected within the sampling extent used. The *nugget effect* (sometimes referred to simply as "nugget") is the amount of variability that is predicted at a separation distance of zero. It represents variance that cannot be modeled using the spatial autocorrelation function, and may occur because of random sampling variance, experimental error, or variance at other spatial scales. When the exponential model was applied to a variogram, estimates were obtained of each of these three parameters. However, when a linear model was used to describe the spatial structure, it was not possible to estimate the range (except to say that it is larger than the analytical extent used to construct the variogram) or the spatial dependence (because the sill is not known).

8.2.5.2.2. Variogram analysis of microbiological data

Because of the relatively small number of samples examined with AODC (N = 59) and T-RFLP (N = 10), analysis of the spatial structure of these parameters was not performed at all analytical scales. For the T-RFLP, only plot-scale variogram analysis was performed; dissimilarity was plotted versus spatial separation to produce a pseudovariogram similar to those used with the AFLP fingerprinting data (Franklin and Mills, 2003). For the AODC data, analyses were performed at both the plot scale and the large scale as described above.

8.2.5.2.3. Kriging

Because the shape of the spatial autocorrelation structure function does not always clearly correspond to a single type of spatial structure, maps were created to represent the spatial distribution of each property using kriging. Variogram models of the original dataset provided the basis for this estimation. Ordinary block kriging was used to produce maps of the distribution of each environmental parameter, and bacterial abundance, at the plot scale, using the SADA statistical package (Spatial Analysis and Decision Assistance, Version 3.0.80, University of Tennessee).

8.2.5.3. Modeling the environment–microbial community relationship

8.2.5.3.1. Approach and data preparation

Causal modeling using Mantel and partial Mantel tests is a way of *interpreting* correlation coefficients in terms of hypothesized causal relationships among variables (Legendre and Legendre, 1998; Legendre and Troussellier, 1988). In this study, it was used to examine the relationship between the microbial communities, the soil microenvironment, and spatial separation, with the overall goal of determining what factors might be important in controlling community composition and abundance. The analysis was performed at each analytical scale (plot, large, small, and fine) to study how the perception/detection of these relationships changed when different spatial extents were considered.

In order to simplify the analyses, the environmental variables were separated into three subgroups: overall soil texture (combining sand, silt, and clay measurements), soil carbon and nitrogen (CN), and organic matter content. Organic matter content was

maintained as a separate variable based on the fact that it did not correlate significantly with any of the other soil properties that were considered (results of a Pearson correlation analysis (not presented), p always greater than 0.1). The relationship of each of these macrovariables (texture, CN, or OM) to either bacterial abundance (AODC) or similarity in microbial community structure (as determined by AFLP DNA fingerprinting) was examined. The T-RFLP results were not considered in this analysis because of the small amount of available data.

Since Mantel tests are used to compare distance/dissimilarity matrices, Gower's coefficient was used to quantify the resemblance among samples (Gower, 1971; Legendre and Legendre, 1998); separate similarity matrices were developed for each set of environmental variables and for bacterial abundance. Geographic (Euclidean) distance was computed for all pairs of sampling locations to assemble a spatial distance matrix. For the community structure data, relative similarity values were calculated using the Jaccard coefficient as previously described (Franklin and Mills, 2003). When necessary, similarity matrices were transformed to dissimilarity matrices as: Dissimilarity = 1 – Similarity.

In addition to studying the relationship of the environmental variables with *overall* community structure, we also wanted to examine whether different subsets of the microbial community responded to different environmental variables. As suggested by Legendre (1993), a principal components analysis (PCA) was used to reduce the multivariate community data (the AFLP patterns) into a set of derived variables describing different aspects of community structure. Each of the resulting principal components (PCs) correlates with a distinct pattern of variability, manifest with different

groups of AFLP bands. Together, these components explained 27 % of the total variance in community structure (PC 1: 13%, PC 2: 8 %, and PC 3: 6 %). Conceptually, these PCs may be considered to represent different "subsets" of the communities' overall genetic composition, and are likely related to the distribution and relative abundance of different populations or groups of organisms. Spatial autocorrelation analysis was performed, and maps of the spatial distribution of each PC were presented in Franklin and Mills (2003). In the present study, causal modeling was performed separately for each community subset (each PC) using similarity matrices obtained with Gower's coefficient.

8.2.5.3.2. Development of conceptual models

In constructing the causal models, the following relationships were hypothesized: (1) space → texture (the patterns observed in soil texture were "caused" by spatial structure (e.g., spatial autocorrelation)), (2) space → OM, (3) space → CN, (4) space → community (either total abundance or community structure), (5) texture → community, (6) OM ↔ community, and (7) CN ↔ community. Because of the strong reciprocal relationship that may exist between microorganisms and the local availability of C, N, and OM, these relationships are presented with bi-directional arrows. In contrast, the microbial community was not expected to significantly influence the particle size distribution of the surrounding soil, at the scales considered here, and so the arrow between "texture" and "community" was drawn in only one direction. Using these

hypothesized relationships, it was possible to construct several possible models of relationships among the variables "space", "environment" (either texture, CN or OM), and "community" (either community structure or abundance) (Figure 8.2).

8.2.5.3.3. Causal modeling

Before presenting the details of this method, it is necessary to briefly describe the Mantel and partial Mantel tests. A Mantel test is a regression in which the variables are actually similarity or distance matrices summarizing pairwise comparisons among samples; the Mantel statistic (r_M) is computed by determining the sum of the cross-products of the corresponding values in each of these matrices (Rossi, 1996). The partial Mantel test, as developed by Smouse et al. (1986), allows testing for the correlation between two matrices while controlling for the effect of a third matrix, and is analogous to a partial correlation. By comparing matrices in this way, it is possible to address questions such as: (i) "do samples that are similar in environment tend to have similar microbial communities?" (Mantel test between a matrix of environmental similarity and a matrix of community similarity); (ii) "do samples that are close together have similar environmental properties?" (Mantel test with a matrix of geographic distances and a matrix of environmental similarity); and (iii) "is there a relationship between community similarity and environmental similarity, after removing the shared correlation of these variables with spatial separation?" (partial Mantel test using a matrix of community similarity and a matrix of environmental similarity, controlling for the effect of a third matrix of geographic spatial separation distances).

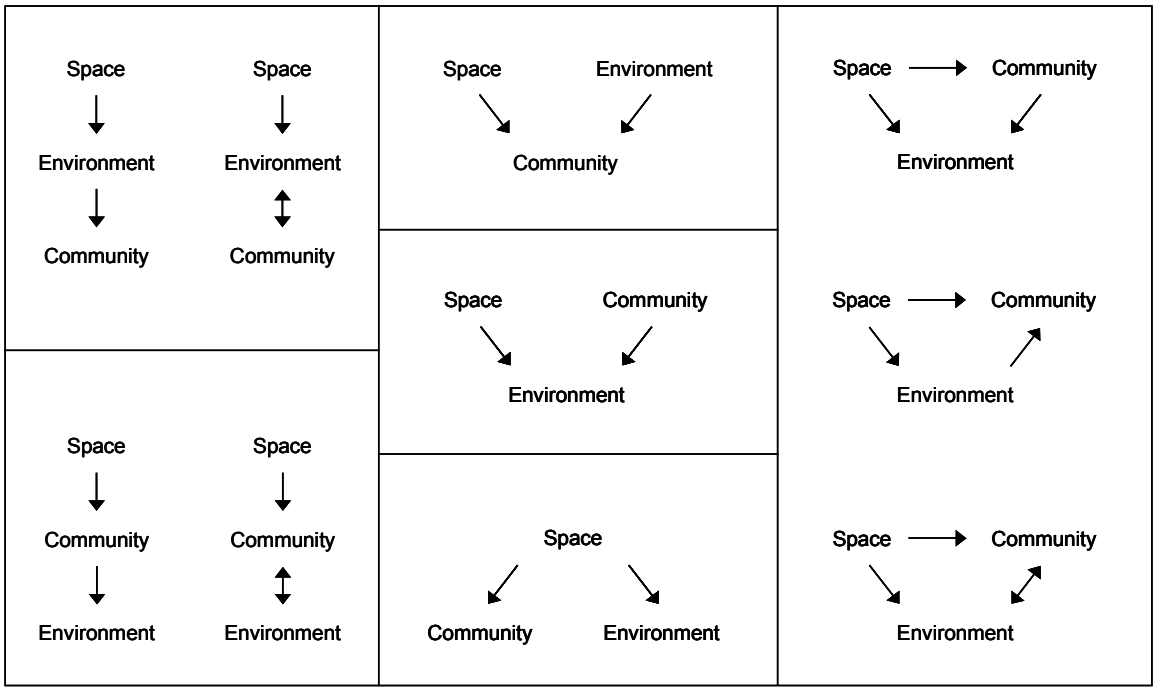

Figure 8.2. Potential individual models among space and the community and environmental variables. All of the models within a particular box are mathematically equivalent, and can only be distinguished based on the hypothesized relationships among variables.

For simple causal modeling, a set of hypotheses is developed about the relationships among three variables; in our case, the variables were: “community”, “space”, and “environment”. A limited number of “models” exist that can be used to represent the relationships among these descriptors (Figure 8.2), and each model corresponds to a set of specific predictions regarding the magnitude and significance of the Mantel and partial Mantel test results. The development of these models and predictions are beyond the scope of this document; Legendre (1993) provides the expectations for all possible causal models involving three matrices in terms of the three simple and three partial Mantel test values.

In this study, normalized Mantel and partial Mantel statistics were calculated to test several hypothetical relationships between the microbial community, soil environment, and spatial structure using the R statistical package (Legendre and Vaudor, 1991). The results (r_M values) range from –1 and + 1, and the statistical significance of each test was evaluated through permutation (a Monte Carlo approach, with 1000 permutations). The significance level was corrected for multiple comparisons using a Bonferroni approach. For each overall community model that was developed, 40 simultaneous tests were performed, and a corrected significance level of 0.001 was used (0.05 / 40 comparisons = 0.001). It is important to point out that, though these Mantel statistics are conceptually similar to Pearson’s correlation or partial correlation coefficients, the magnitude of a Mantel or partial Mantel statistic is often small, even when highly statistically significant (Dutilleul et al., 2000).

For each combination of an environmental variable and a community variable, all possible causal models were evaluated by comparing the Mantel and partial Mantel test results with the predictions outlined in Legendre (1993). Individual models were rejected when the results did not conform to these predications, and, by process of elimination, a single plausible model was derived for each relationship. For each scale, these individual models were pooled into a single integrated model summarizing all of the observed relationships in the system (Legendre and Troussellier, 1988).

This modeling approach is based on correlation, and finding a significant relationship between two variables does not actually demonstrate cause. None-the-less, *failing* to find a relationship between two variables suggests against a causal relationship. In this context, "causality" refers to the hypothesis that changes occurring in one variable have an effect on changes in another variable; data are said to support the causality hypothesis if a significant portion of the variation in one property in explained by changes taking place in the second property (Legendre and Legendre, 1998). It should be noted that the correlation between two distance matrices in not equivalent to the correlation between the two variables behind these matrices; the matrix correlation specifically measures the extent to which the variations in the distances of Matrix A correspond to variations in Matrix B (Dutilleul et al., 2000).

8.3. Results

8.3.1. Characterization of spatial structure

8.3.1.1. Variogram analysis of environmental data

At the plot scale, spatial autocorrelation was observed for all of the environmental variables (Table 8.2). In general, there was a good fit of the models to the data (average $R^2 = 0.72$, range: 0.50 to 0.91), with very low p values. Most often, a linear relationship was observed in the variograms (Figure 8.3), which indicates significant spatial autocorrelation over the entire sampling extent, and so no range estimate could be made. An exponential model was applied to the N and OM variograms, and range values of 5.4 m and 6.2 m were calculated, respectively.

Variogram analysis at other analytical scales indicated a large amount of autocorrelation structure, nested within the patterns observed for the entire plot (Table 8.2, variograms not presented). R^2 values were generally high (average R^2: large scale – 0.72, small scale – 0.59, and fine scale – 0.66), and similar to those obtained for the plot-scale analysis. For sand, silt, and OM content, a linear spatial pattern was observed at both the large and small scales, and no structure was detected at the fine scale. For the other variables, multiple scales of spatial autocorrelation, with nested CLS, were detected (Table 8.2, Figure 8.4). Specifically, patch sizes (range estimates) for C were 0.7 m and 3.7 m, and additional autocorrelation was observed at separation distances up to 11 m. Similar results were obtained in the analysis of clay (patch sizes: 2.5 m and > 11 m) and N (patch sizes: 0.2 m, 0.8 m, and 5.4 m).

Table 8.2. Summary of geostatistics for each scale. Only statistically significant model fits are reported ($p < 0.05$), all others are labeled as 'nugget'.

Parameter	Model type	R^2	p	Nugget	Spatial dependence	Range (m)
Plot-scale (≤ 11 m)						
AFLP *	Exponential	0.67	0.001	0.68	0.14	6.3
Carbon	Linear	0.91	< 0.0001	0.13		> 11
Nitrogen	Exponential	0.63	0.0006	0.33	0.59	5.4
OM	Exponential	0.57	0.01	0.45	0.48	6.2
Sand	Linear	0.90	< 0.0001	0.00		> 11
Silt	Linear	0.81	< 0.0001	0.01		> 11
Clay	Linear	0.50	0.0003	0.41		> 11
Large-scale (≤ 5 m)						
AFLP *	Exponential	0.70	< 0.0001	0.66	0.15	2.0
Carbon	Exponential	0.85	0.0002	0.35	0.63	3.7
Nitrogen	Linear	0.58	0.003	0.42		> 5.0
OM	Linear	0.53	0.02	0.51		> 5.0
Sand	Linear	0.87	< 0.0001	0.15		> 5.0
Silt	Linear	0.69	0.001	0.34		> 5.0
Clay	Exponential	0.82	0.006	0.48	0.85	2.5
Small-scale (≤ 1 m)						
AFLP *	Linear	0.83	< 0.0001	0.66		> 1.0
Carbon	Exponential	0.70	0.03	0.41	0.54	0.7
Nitrogen	Exponential	0.74	0.009	0.49	0.50	0.8
OM	Linear	0.64	0.003	0.68		> 1.0
Sand	Linear	0.11	0.04	0.38		> 1.0
Silt	Linear	0.71	0.002	0.33		> 1.0
Clay	Linear	0.63	< 0.0001	0.31		> 1.0
Fine-scale (≤ 0.4 m)						
AFLP *	Linear	0.52	0.003	0.67		> 0.4
Carbon	Nugget					
Nitrogen	Exponential	0.79	< 0.0001	0.25	0.73	0.2
OM	Nugget					
Sand	Nugget					
Silt	Nugget					
Clay	Nugget					

* Franklin and Mills (2003), based on dissimilarity matrices.

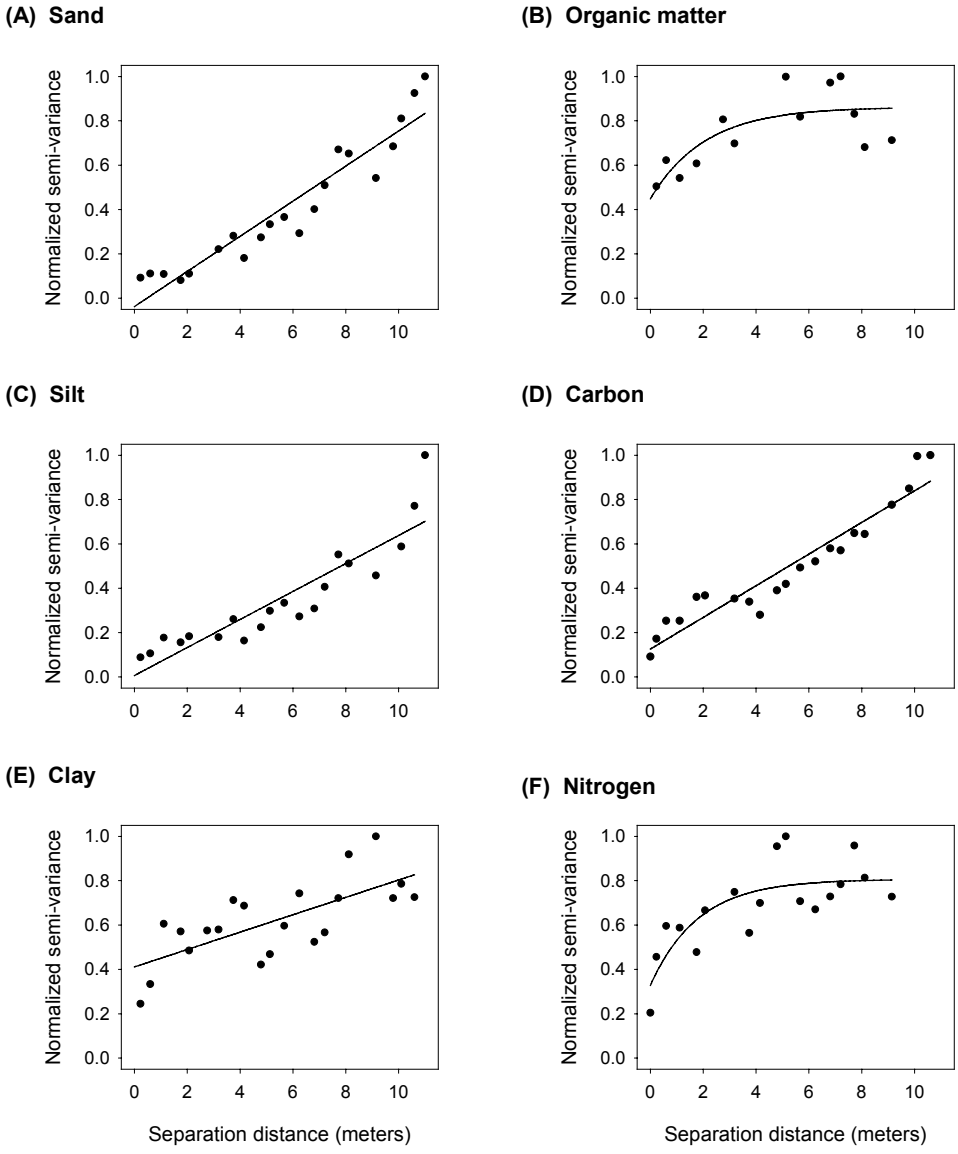

Figure 8.3. Plot-scale variograms for environmental properties.

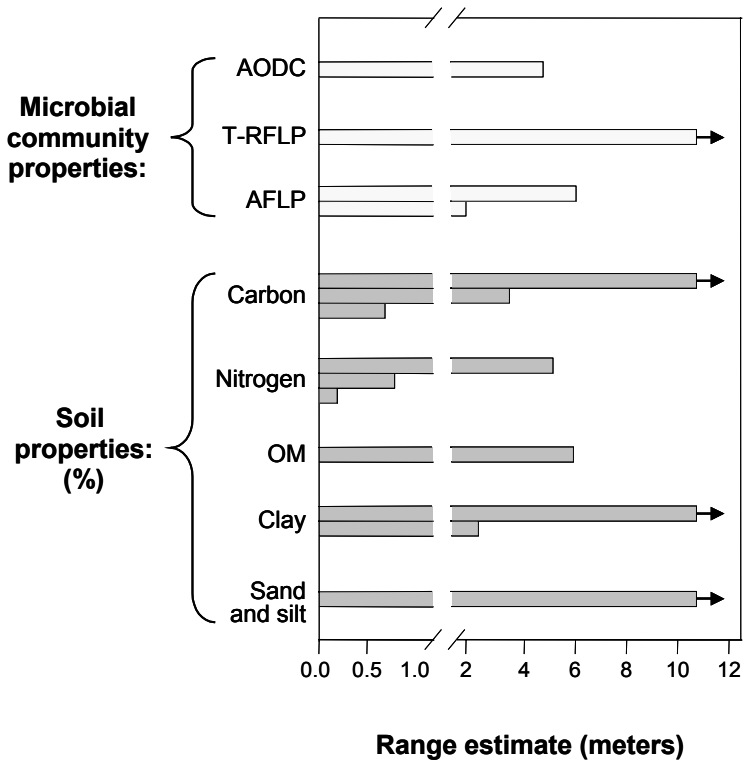

Figure 8.4. Summary of range estimates from the variogram analyses of the microbial and environmental properties at the different analytical scales. The placement of an arrow at the end of a bar denotes that the range for that variable could not be determined, but is known to be "greater than" the number indicated.

Spatial dependence was calculated for those variables modeled using the exponential equation. In general, the spatial autocorrelation model explained slightly more than half of the variability observed in those analyses (range: 0.48 – 0.85, average: 0.62). These values were similar for the different variables and across the four analytical scales, but much higher than those obtained in the previous analysis of microbial community structure (Table 8.2).

8.3.1.2. Variogram analysis of microbiological data

At the plot scale, the T-RFLP results indicate the presence of spatial autocorrelation in community structure based on variability in the 16S rRNA gene (Figure 8.5 C). Considering the small number of samples analyzed (N = 45 pairwise comparisons), caution must be used when interpreting these results; nonetheless, an increasing trend is visible (linear fit, $R^2 = 0.30$, $p < 0.0001$). Samples collected from the same node were quite similar, and distinct from those collected at other nodes (Figure 8.5 D).

Overall, 40 different T-RF were observed across all of the T-RFLP fingerprints, and 10 of these fragments (25%) were common to all samples. For the samples collected at node A, several unique fragments were observed (up to 7), while samples from other nodes contained few, if any, unique markers. The fingerprints generated from the samples collected at node A also contained a higher number of fragments (average for node A = 26.5, average for all other samples = 18), compared to the other samples.

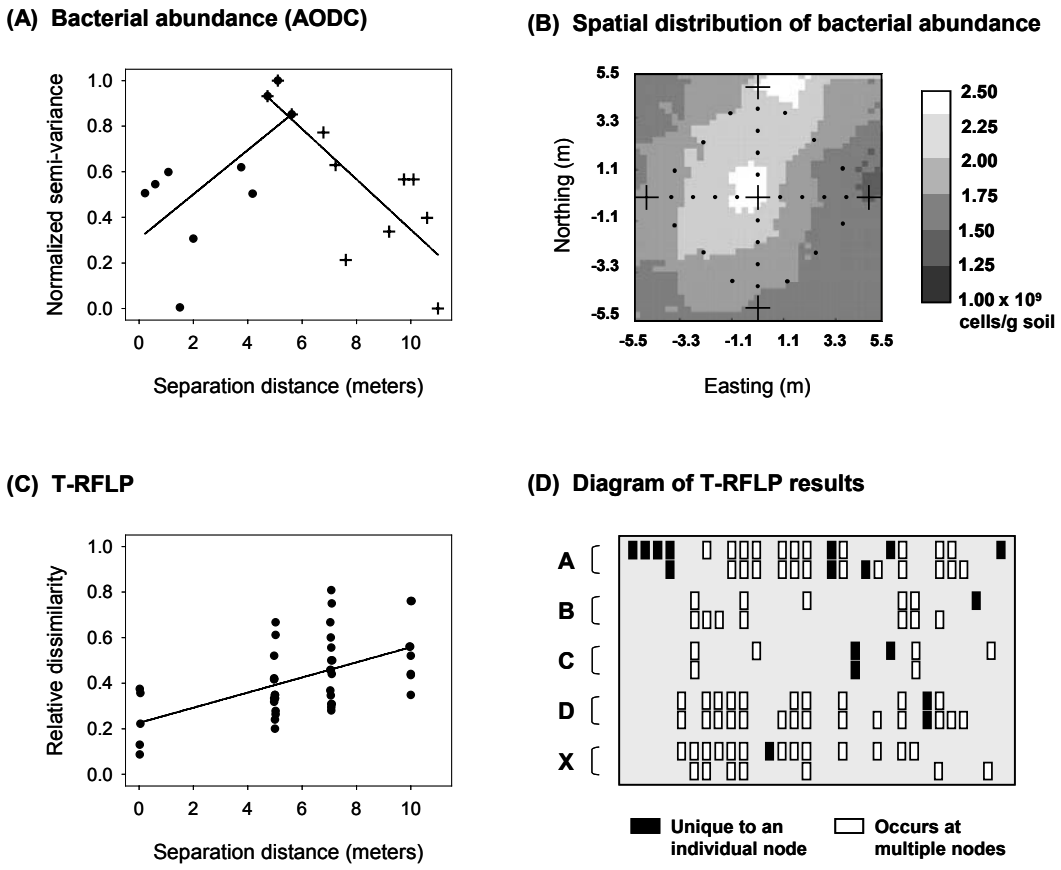

Figure 8.5. Results of AODC and T-RFLP analysis. (A) Plot-scale variogram for bacterial abundance data. (B) Kriged map showing the spatial distribution of bacterial abundance across the plot. The circular symbols are used for Section 1, and the crosses are used for Section 2 (see text). (C) Plot-scale variogram for T-RFLP data. (D) Results of the T-RFLP, presented in a diagram indicating which restriction fragments (bands) were present in each individual sample. Ten additional markers, present in all of the samples, were obtained, but omitted from the figure for simplicity.

The bacterial abundance data displayed neither a linear nor an exponential trend at the plot scale (N = 1711 pairwise comparisons) (Figure 8.5 A). Instead, the graph had a peaked pattern, where abundance values became more different as separation distances increased, until approximately 5 – 6 m, and then became more similar at larger separation distances. This pattern is consistent with variograms obtained when analyzing a circular gradient. The magnitude of the slopes of the ascending and descending halves of the variogram were nearly identical (Section 1 (0 – 6 m): $R^2 = 0.43$, $p = 0.04$, slope = 0.10; Section 2 (5 – 11 m): $R^2 = 0.65$, $p = 0.003$, slope = - 0.11), which suggests that the rate of change of the microbial community with distance was approximately equal in each direction away from the center of the patch. The results for the analysis of the ascending half of the plot-scale variogram (≤ 6 m) are essentially the same as those obtained for the large-scale analysis (≤ 5 m, N = 595 pairwise comparisons): linear model, $R^2 = 0.62$, $p = 0.06$, nugget = 0.37, and range > 5 m.

8.3.1.3. Kriging results

Ordinary kriging was used to generate maps of the spatial distribution of each property at the plot scale (Figure 8.5 B and Figure 8.6). Most of the environmental variables presented a gradient-like distribution over the sampling area, extending from the NE corner (upper right) of the plot across to the SW corner (lower left). This is particularly true for the variables that only displayed larger-scale linear patterns of spatial autocorrelation in the variogram analysis (e.g., sand and silt). For carbon, nitrogen, and,

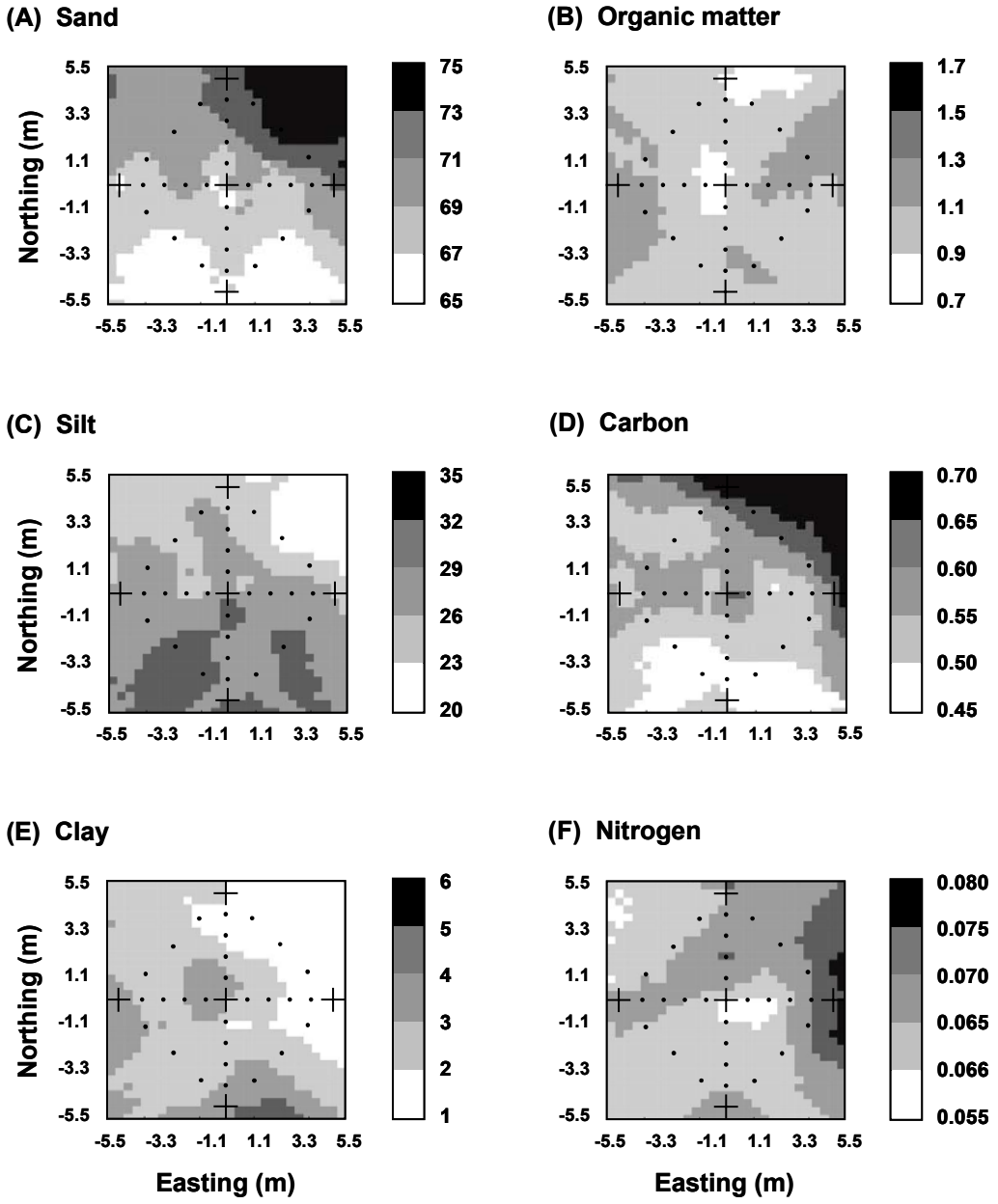

Figure 8.6. Kriged maps showing the distribution of each environmental variable at the plot scale. The maps are oriented to match Figure 8.1A. Sampling nodes are indicated with '+', and the perimeter and diagonal sampling locations are marked with '•'. The legend included with each map has units of 'percent'.

to a lesser extent, clay, smaller-scale spatial heterogeneity is also visible within the larger-scale structure. This is consistent with the results from the variogram analysis, where C, N, and clay were the only environmental variables that displayed multiple CLS (Figure 8.4).

For bacterial abundance, kriging produced a map with a bulls-eye pattern, focused around the center of the plot (Figure 8.5 B). Because of the unusual shape of the variogram obtained for AODC, the variogram model used to create this map was limited to separation distances less than 6 m (the ascending portion of the curve). A second map was also produced using an inverse distance/nearest neighbor type algorithm (results not presented), and the pattern observed was quite similar to the original kriged map; this finding helps to confirm the validity of the kriged map.

On some of the kriged maps (e.g., OM), aberrations or patches were occasionally observed in association with the individual sampling nodes (A – X). To address the concern that this phenomena could be an artifact, generated by the high sample density at these locations, another set of maps were created using a single, averaged value to represent each node (results not presented). These maps did not differ much from those presented in Figure 8.6.

8.3.2. Causal modeling

8.3.2.1. Overall community structure

Causal models considering overall community structure (similarity calculated from the complete AFLP dataset) and the relationships among the different environmental variables were produced for each analytical scale (Figure 8.7, Tables 8.3

and 8.4). The models generated for the plot and large scales were the same for this comparison, and spatial separation was significantly correlated with the distribution of all of the environmental variables and microbial community structure (AFLP). A strong correlation between soil texture and CN content was also observed. None of the environmental variables were shown to correlate directly with overall community structure.

The results for the analysis at the small scale were similar to those obtained for the plot and large scales. However, other relationships between the environmental variables were observed; in particular, changes in OM content were found to be correlated with changes in soil texture and CN. For the fine scale, fewer significant relationships were observed, and spatial separation was shown to be only weakly correlated with community structure and soil texture.

8.3.2.2. Individual community subsets

Causal models were also produced to examine the relationship between each community subset (each PC) and the spatial and environmental variables (Figure 8.8). In order to simplify these diagrams, the relationships *among* the environmental variables not directly correlated with community structure were not presented (already included in Figure 8.7).

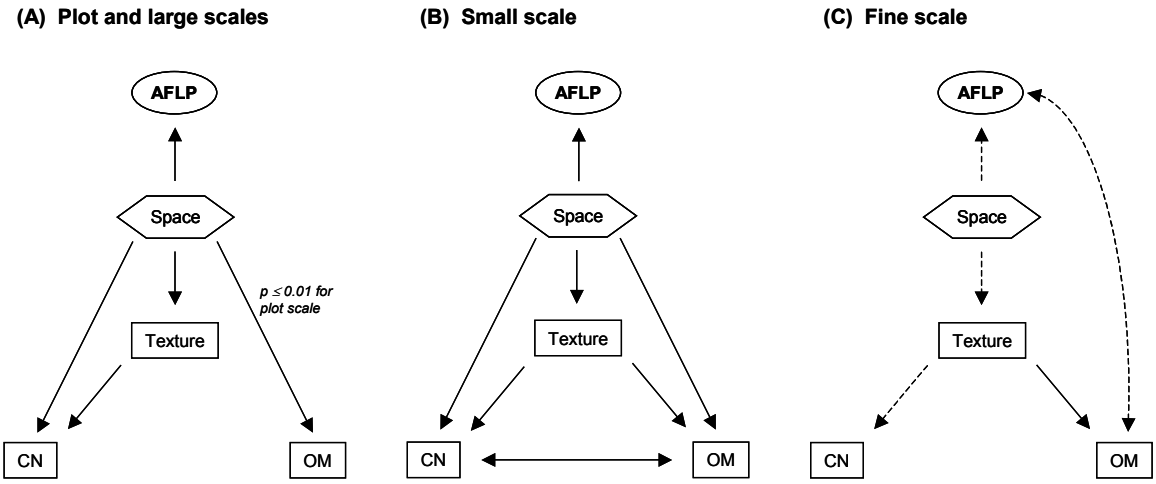

Figure 8.7. Causal models depicting the relationship among space and the different environmental variables, and between these variables and overall community structure (AFLP profiles), at each analytical scale. Each solid line is significant with p values ≤ 0.001; dashed lines correspond to nearly significant results with p values $0.001 < p \leq 0.01$.

Table 8.3. Mantel and partial Mantel test results examining the relationship among space and each environmental variable. The "partial effect variable" indicates the third matrix used in the partial Mantel tests (the shared correlation of this variable with each main variable has been removed). A partial effect variable of "none" indicates a direct Mantel test. Mantel statistics (r_M) are presented, and statistically significant values are indicated.

Main Variables		Partial effect variable	Scale			
A	**B**		Plot	Large	Small	Fine
Space	CN	None	- 0.31 *	- 0.39 *	- 0.15 *	- 0.04
		OM	- 0.30 *	- 0.39 *	- 0.14 *	- 0.04
		Texture	- 0.19 *	- 0.34 *	- 0.14 *	- 0.04
		AFLP	- 0.30 *	- 0.38 *	- 0.16 *	- 0.04
		AFLP PC1	- 0.30 *	- 0.39 *	- 0.15 *	- 0.04
		AFLP PC 2	- 0.30 *	- 0.39 *	- 0.14 *	- 0.04
		AFLP PC 3	- 0.24 *	- 0.37 *	- 0.15 *	- 0.04
	OM	None	- 0.05 +	- 0.11 *	- 0.09 *	- 0.02
		CN	- 0.04	- 0.11 *	- 0.08 *	- 0.02
		Texture	- 0.06 +	- 0.12 *	- 0.08 *	- 0.02
		AFLP	- 0.05 +	- 0.11 *	- 0.08 *	- 0.02
		AFLP PC1	- 0.05 +	- 0.11 *	- 0.09 *	- 0.02
		AFLP PC 2	- 0.05 +	- 0.08 *	- 0.09 *	- 0.02
		AFLP PC 3	- 0.07 +	- 0.13 *	- 0.09 *	- 0.02
	Texture	None	- 0.42 *	- 0.34 *	-0.12 *	- 0.05 +
		CN	- 0.36 *	- 0.30 *	- 0.11 *	- 0.06 +
		OM	- 0.42 *	- 0.34 *	- 0.11 *	- 0.05 +
		AFLP	- 0.40 *	- 0.34 *	-0.13 *	- 0.05 +
		AFLP PC1	- 0.42 *	- 0.34 *	-0.12 *	- 0.05 +
		AFLP PC 2	- 0.42 *	- 0.34 *	-0.12 *	- 0.05 +
		AFLP PC 3	- 0.37 *	- 0.31 *	-0.12 *	- 0.05 +

* $p \leq 0.001$
+ $0.001 < p \leq 0.01$

Table 8.3. *Continued*

Main Variables		Partial effect variable	Scale			
A	**B**		Plot	Large	Small	Fine
CN	OM	None	0.04	0.05 *	0.12 *	0.05
		Space	0.03	0.03	0.11 *	0.05
		Texture	0.05	0.06 *	0.11 *	0.04
		AFLP	0.04	0.05 *	0.12 *	0.04
		AFLP PC1	0.05	0.06 *	0.12 *	0.03
		AFLP PC 2	0.04	0.03 +	0.11 *	0.03
		AFLP PC 3	0.05	0.06 *	0.12 *	0.04
	Texture	None	0.33 *	0.24 *	0.10 *	0.07 +
		Space	0.23 *	0.14 *	0.09 *	0.08 +
		OM	0.33 *	0.24 *	0.11 *	0.07 +
		AFLP	0.32 *	0.23 *	0.11 *	0.07 +
		AFLP PC1	0.33 *	0.24 *	0.12 *	0.07 +
		AFLP PC 2	0.32 *	0.23 *	0.10 *	0.06 +
		AFLP PC 3	0.29 *	0.22 *	0.11 *	0.06 +
OM	Texture	None	-0.01	0.002	0.10 *	0.09 *
		Space	-0.03	-0.04 +	0.09 *	0.09 *
		CN	-0.02	-0.01	0.09 *	0.08 *
		AFLP	-0.01	0.00	0.10 *	0.09 *
		AFLP PC1	-0.01	0.00	0.10 *	0.09 *
		AFLP PC 2	-0.01	-0.01	0.10 *	0.08 *
		AFLP PC 3	0.00	0.01	0.10 *	0.08 *

* $p \leq 0.001$
+ $0.001 < p \leq 0.01$

Table 8.4. Mantel and partial Mantel test results examining the relationship of space and each environmental variable with community structure. The "partial effect" variable indicates the third matrix in the partial Mantel test (the shared correlation of this variable with each main variable has been removed). A partial effect of "none" indicates a direct Mantel test. Mantel statistics (r_M) are presented, and statistically significant values are indicated.

Variable	Scale	Partial effect	AFLP	AFLP PC 1	AFLP PC 2	AFLP PC 3
Space	Plot	None	- 0.26 *	- 0.02	- 0.10 *	- 0.38 *
		CN	- 0.25 *	0.03	- 0.08 *	- 0.34 *
		OM	- 0.26 *	- 0.02	- 0.09 *	- 0.38 *
		Texture	- 0.26 *	- 0.02	- 0.08 *	- 0.32 *
	Large	None	- 0.22 *	- 0.03	- 0.22 *	- 0.23 *
		CN	- 0.21 *	0.02	- 0.20 *	- 0.20 *
		OM	- 0.22 *	- 0.03 +	- 0.21 *	- 0.24 *
		Texture	- 0.22 *	- 0.02	- 0.22 *	- 0.19 *
	Small	None	- 0.12 *	- 0.05 +	- 0.03	- 0.01
		CN	- 0.13 *	- 0.04	- 0.01	0.02
		OM	- 0.12 *	0.03	- 0.02	0.00
		Texture	- 0.13 *	0.04	- 0.02	0.00
	Fine	None	- 0.06 +	- 0.04	- 0.01	- 0.04
		CN	- 0.06 +	- 0.04	- 0.02	- 0.03
		OM	- 0.06 +	- 0.03	- 0.01	- 0.04
		Texture	- 0.06 +	- 0.04	- 0.01	- 0.04
CN	Plot	None	0.06	0.13 *	0.11 *	0.21 *
		Space	- 0.04	0.14 *	0.07 *	0.11 *
		OM	0.02	0.14 *	0.10 *	0.22 *
		Texture	0.00	0.14 *	0.09 *	0.16 *
	Large	None	0.06	0.12 *	0.19 *	0.12 *
		Space	0.00	0.12 *	0.11 *	0.05 *
		OM	0.07	0.12 *	0.18 *	0.12 *
		Texture	0.06	0.12 *	0.18 *	0.09 *
	Small	None	0.00	0.06 *	0.15 *	0.13 *
		Space	- 0.02	0.06 *	0.14 *	0.13 *
		OM	- 0.01	0.06 *	0.15 *	0.13 *
		Texture	0.00	0.06 *	0.14 *	0.12 *
	Fine	None	0.04	0.03	0.21 *	0.16 *
		Space	0.04	0.03	0.21 *	0.16 *
		OM	0.04	0.03	0.21 *	0.16 *
		Texture	0.04	0.03	0.21 *	0.15 *

* $p \leq 0.001$, + $0.001 < p \leq 0.01$

Table 8.4. *Continued*

Variable	Scale	Partial effect	AFLP		AFLP PC 1		AFLP PC 2		AFLP PC 3	
OM	Plot	None	0.01		- 0.05		0.09	+	- 0.02	
		Space	0.001		- 0.05		0.09	*	- 0.05	
		CN	0.01		- 0.05		0.09	*	- 0.03	
		Texture	0.02		- 0.05		0.09	*	0.02	
	Large	None	0.04	*	- 0.04		0.16	*	- 0.05	
		Space	0.02		- 0.05		0.14	*	- 0.08	+
		CN	0.04	+	- 0.05		0.16	*	- 0.05	
		Texture	0.04	+	- 0.04		0.16	*	- 0.05	
	Small	None	0.03		0.03		0.05	+	0.00	
		Space	0.02		0.02		0.05	+	0.00	
		CN	0.03		0.03		0.05	+	- 0.01	
		Texture	0.03		0.03		0.05	+	- 0.01	
	Fine	None	0.06	+	0.05		0.08	*	0.04	
		Space	0.06	+	0.05	+	0.08	*	0.04	
		CN	0.06	+	0.05		0.07	*	0.03	
		Texture	0.06	+	0.05		0.07	*	0.03	
Texture	Plot	None	- 0.07		0.05		0.06	*	0.22	*
		Space	- 0.06		- 0.01		0.02		0.07	*
		CN	0.16	*	- 0.04		0.03		0.22	*
		OM	0.18	*	- 0.04		0.03		0.18	*
	Large	None	0.05		0.03		0.06	*	0.16	*
		Space	- 0.02		- 0.02		- 0.02		0.09	*
		CN	0.04	+	0.00		0.02		0.14	*
		OM	0.05	*	0.03		0.06	*	0.16	*
	Small	None	- 0.02		0.01		0.06	+	0.08	*
		Space	- 0.04		0.00		0.04		0.08	*
		CN	- 0.01		0.01		0.06	+	0.07	*
		OM	- 0.02		0.00		0.05		0.08	*
	Fine	None	- 0.02		- 0.01		0.06	+	0.08	*
		Space	- 0.01		- 0.01		0.04		0.08	*
		CN	- 0.02		- 0.01		0.05	+	0.07	+
		OM	- 0.02		- 0.01		0.04		0.07	+

* $p \leq 0.001$, $^{+}$ $0.001 < p \leq 0.01$

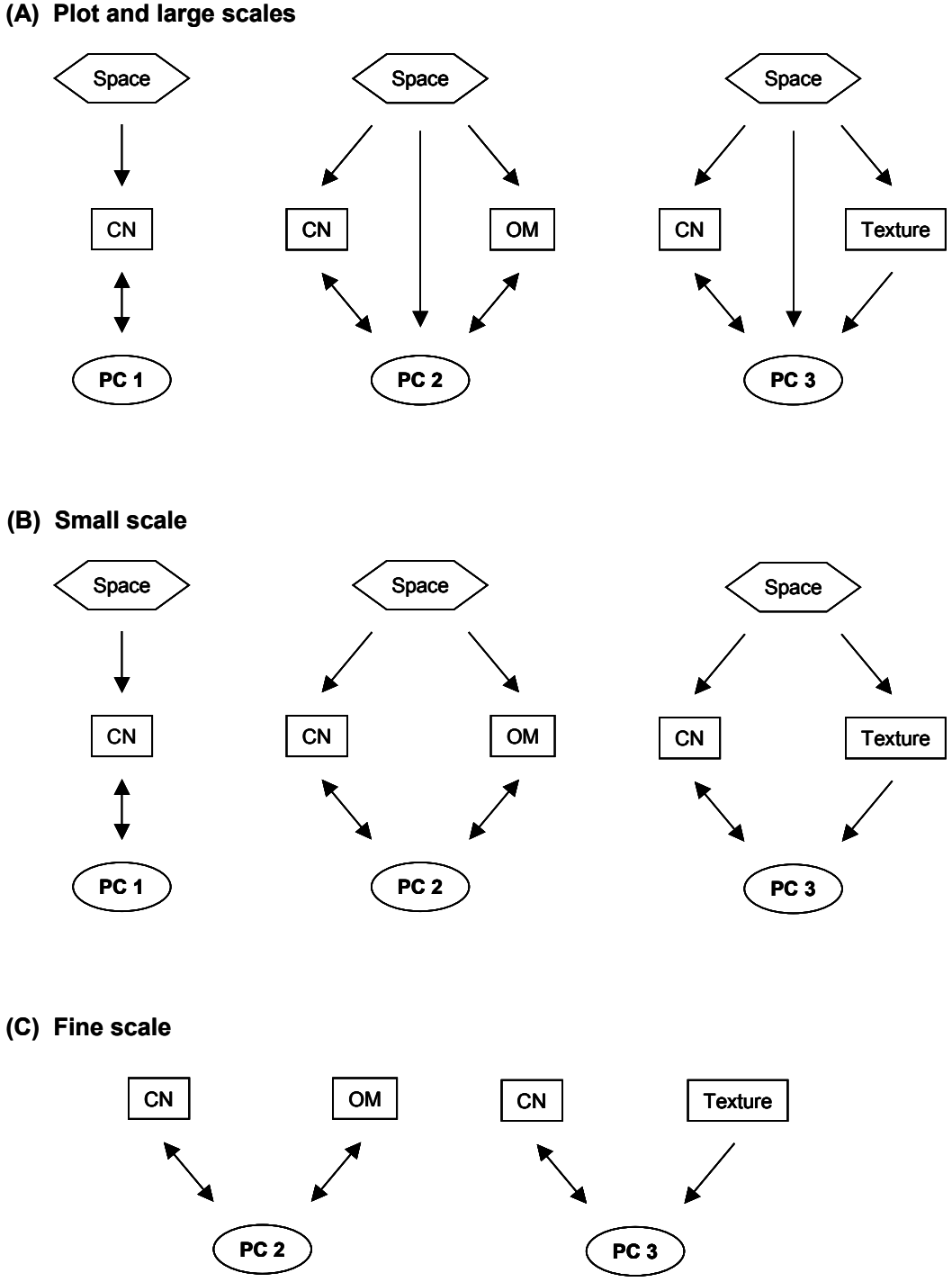

Figure 8.8. Causal models depicting the relationship among space, the environmental variables, and each subset of the microbial community (each PC), at each analytical scale. For simplicity, these diagrams only show the variables that were directly correlated with community structure; relationships among the various environmental properties are not presented (see Figure 8.7). The impact of space on each model is also included. Each solid line is significant with p values ≤ 0.001.

As was found for overall community structure, the models generated at the plot and large scales were identical. Changes in community subset PC 1 were significantly correlated with changes in soil CN at separations distances larger than 1 m (plot-, large-, and small-scale analyses), but not at the finest scale. Changes in community subset PC 2 were associated with changes in both CN and OM at all of the scales considered. However, the relationship of spatial separation to changes in this subset of the microbial community was different at different analytical scales. At both the plot and large scales, spatial separation was directly correlated to changes in PC 2, and to changes in both of the environmental variables controlling PC 2 (CN and OM). At the small scale, the direct influence of space on PC 2 was not present, though space was still indirectly connected to PC 2 through the controlling environmental variables (CN and OM). At the fine scale, CN and OM were again correlated with PC 2, but no significant relationships with space were observed. For the different analytical scales, the same results were obtained for community subset PC 3, except that the relationship with OM was replaced by a relationship with soil texture. The decreasing importance of spatial separation on community structure was again observed.

8.3.2.3. Bacterial abundance

Because of the relatively small number of samples analyzed with AODC, modeling was only performed at the plot and large scales (Figure 8.9, Table 8.5). In both cases, OM was the only environmental variable that correlated with bacterial abundance. Spatial separation was also found to be indirectly correlated with changes in bacterial

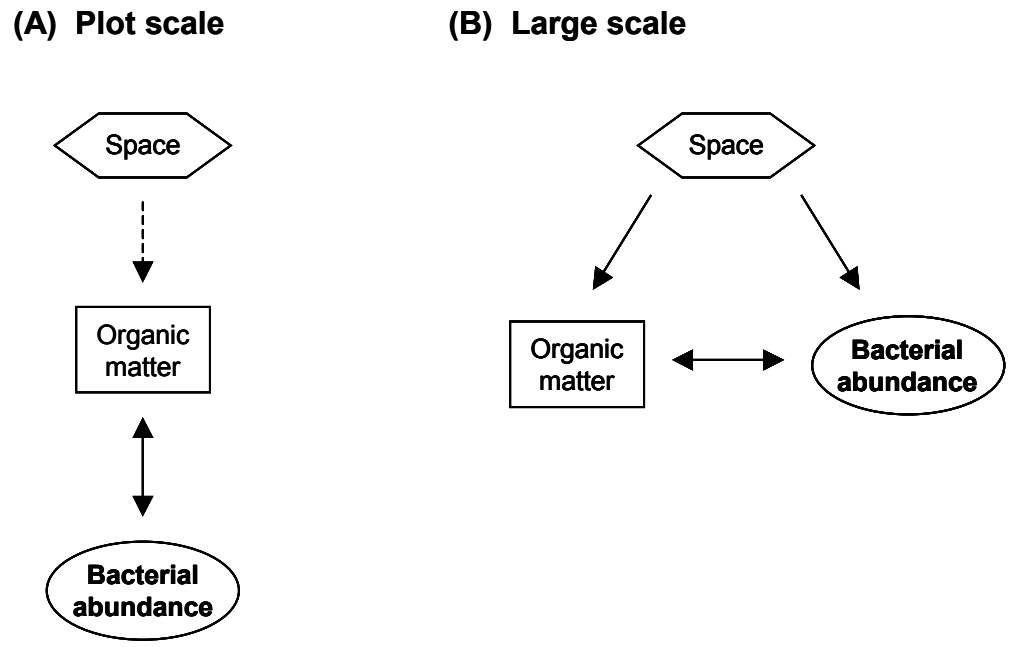

Figure 8.9. Causal models depicting the relationship among space, the environmental variables, and bacterial abundance (AODC), at each analytical scale. Only the environmental variables that were directly correlated with bacterial abundance are presented, relationships among environmental variables are not repeated (see Figure 8.7). The impact of space on each model is also included. Each solid line is significant with p values ≤ 0.001; dashed lines correspond to p values $0.001 < p \leq 0.01$.

Table 8.5. Mantel and partial Mantel test results examining the relationship of space and each environmental variable with total bacterial abundance (AODC). The "partial effect variable" indicates the third matrix used in the partial Mantel tests (the shared correlation of this variable with each main variable has been removed). A partial effect variable of "none" indicates a direct Mantel test. Mantel statistics (r_M) are presented, and statistically significant values are indicated.

Plot scale (up to 11 meters)						
Variables		**Second main effect variable**				
Main effect	Partial effect	Space	CN	OM	Texture	AODC
Space	None	–	-0.43 *	- 0.08 +	- 0.51 *	0.02
	CN	–	–	- 0.10 +	- 0.41 *	- 0.01
	OM	–	- 0.43 *	–	- 0.51 *	0.02
	Texture	–	- 0.29 *	- 0.12 +	–	- 0.04
	AODC	–	- 0.43 *	- 0.09 +	- 0.51 *	–
CN	None	- 0.43 *	–	- 0.01	0.40 *	- 0.02
	Space	–	–	- 0.05	0.23 *	- 0.03
	OM	- 0.43 *	–	–	0.40 *	- 0.02
	Texture	- 0.29 *	–	0.01	–	0.00
	AODC	- 0.43 *	–	0.00	0.40 *	–
OM	None	- 0.08 +	- 0.01	–	- 0.05	0.19 *
	Space	–	- 0.05	–	- 0.10	0.19 *
	CN	- 0.10 +	–	–	- 0.05	0.19 *
	Texture	- 0.12 +	0.01	–	–	0.19 *
	AODC	0.09 +	0.00	–	- 0.03	–
Texture	None	- 0.51 *	0.40 *	- 0.05	–	- 0.06
	Space	–	0.23 *	- 0.10	–	- 0.07
	CN	- 0.41 *	–	- 0.05	–	- 0.06
	OM	- 0.51 *	0.40 *	–	–	- 0.06
	AODC	- 0.51 *	0.40 *	- 0.03	–	–

* $p \leq 0.001$, + $0.001 < p \leq 0.01$

Table 8.5. *Continued*

Large scale (up to 5 meters)						
Variables		**Second main effect variable**				
Main effect	Partial effect	Space	CN	OM	Texture	AODC
Space	None	–	- 0.38 *	- 0.36 *	- 0.39 *	- 0.22 *
	CN	–	–	- 0.40 *	- 0.33 *	- 0.21 *
	OM	–	- 0.42 *	–	- 0.47 *	- 0.17 *
	Texture	–	- 0.31 *	- 0.44 *	–	- 0.25 *
	AODC	–	- 0.38 *	- 0.33 *	- 0.41 *	–
CN	None	- 0.38 *	–	0.04	0.27 *	0.05
	Space	–	–	0.02	0.15 *	- 0.03
	OM	- 0.42 *	–	–	0.27 *	0.06
	Texture	- 0.31 *	–	- 0.01	–	0.06
	AODC	- 0.38 *	–	0.04	0.28 *	–
OM	None	- 0.36 *	0.04	–	0.001	0.18 *
	Space	–	0.02	–	- 0.04	0.11 *
	CN	- 0.40 *	–	–	0.02	0.18 *
	Texture	- 0.44 *	- 0.01	–	–	0.18 *
	AODC	- 0.33 *	0.04	–	- 0.01	–
Texture	None	- 0.39 *	0.27 *	0.001	–	- 0.03
	Space	–	0.15 *	- 0.04	–	- 0.13 *
	CN	- 0.33 *	–	0.02	–	- 0.04
	OM	- 0.47 *	0.27 *	–	–	- 0.01
	AODC	- 0.41 *	0.28 *	- 0.01	–	–

* $p \leq 0.001$, + $0.001 < p \leq 0.01$

abundance at the plot scale, and directly correlated to changes in abundance at the large scale. These results are consistent with those obtained with the kriging (similar maps for AODC and OM (Figures 8.5 B and 8.6 B)) and variogram analyses.

8.4. Discussion

This study was designed to address the general need for increased research into multi-scale patterns of spatial organization in soil systems, and the relationship of these patterns to the distribution patterns observed for the soil microbial communities. In particular, the research focused on quantifying the spatial patterns associated with several physical-chemical soil properties and microbial community properties (structure and abundance) in an agricultural field. The results obtained for the two sets of variables were then statistically compared in an effort to better understand the factors that may influence community organization and help constrain bacterial distributions at different spatial scales.

The agricultural field described in this study was originally selected to represent a *relatively* homogeneous system, given that it had been plowed and planted as a single crop for several years. Despite this history, and the small area sampled (50 m^2), a great deal of spatial variability was observed in the physical-chemical properties of the soil. At the plot scale, significant spatial autocorrelation was detected for all of the variables considered (Table 8.2, Figure 8.3); most often, a linear pattern was observed, indicating spatial autocorrelation over the entire sampling extent. However, for OM and N, patch sizes of 5.4 m and 6.2 m, respectively, were calculated.

At smaller analytical scales, additional patterns of spatial autocorrelation were observed for C and N, and for clay content (Table 8.2, Figure 8.4). The presence of these multiple scales of autocorrelation suggests that those variables may display a more patchy distribution, compared to the variables that have a single consistent autocorrelation structure (e.g., sand and silt). This speculation can be confirmed by examining the kriged maps for each soil parameter (Figure 8.6). In general, the maps show a gradient pattern that extends across the plot; this gradient-like distribution suggests the action of some underlying non-random process contributing to the plot-scale spatial structure of the environment in this field. Previous work has shown that compaction due to wheel traffic (Parkin, 1993), or growth in crop rows versus aisles (Ettema and Wardle, 2002; Robertson et al., 1997; Stoyan et al., 2000), may contribute to the development of spatial structure in agricultural fields at similar scales. However, none of these particular land management practices are likely to generate the types of spatial patterns observed here. It is possible that this variation instead corresponds to historical differences in land use across the field (e.g., different boundaries between crops at an earlier time) or to some type of uncharacterized environmental feature (e.g., a small change in topography or in the predominant wind direction). Alternately, the lagoonal shoreline lies approximately 700 m to the east of the plot, and it is also possible that the gradient is somehow associated with proximately to the coast (e.g., increased sand content on the east side of the plot).

The increased spatial variability for C and N, and the presence of patches nested within the overall gradient pattern, could be the result of short-range variations in plant growth, superimposed on the larger-scale patterns discussed above, as the spatial

organization of soil microorganisms has been linked to that of plants several times (Allen and MacMahon, 1985; Klironomos et al., 1999; Robertson et al., 1997; Schlesinger et al., 1996). However, any variability caused by plant growth would be expected to generate similar patches in both the C and N maps; instead, the spatial distribution patterns for these two variables were unique. Because the C and N measures were of the *total* soil pool, they reflect not only spatial heterogeneity associated with biological activity, but may also be influenced by liming and fertilization of the soil. These practices have been shown to directly influence microbial communities, typically resulting in increased biomass and changes in microbial functional properties (Brodie et al., 2003; Lovell and Jarvis, 1998). Recent work by Sarathchandra et al. (2001) has also demonstrated that the addition of N via fertilization may influence microbial community composition and activity.

While significant spatial structure was detected for all of the variables considered at the larger analytical scales, only N and community structure displayed spatial autocorrelation at the fine scale (≤ 0.4 m). Previously, we found that the spatial distribution of community structure at the finer scales was different at the different nodes in the plot, so it is possible that a clear pattern was simply not detected because the data from all of these locations was pooled in the present analysis. If the fine-scale spatial structure of a variable changes across the sampling plot, it may not be possible to observe an overall "average" pattern. The decreased number of samples at the fine scale (N = 1670 pairwise comparisons), compared to the large (N = 8288) and plot (N = 18528) scale analyses, could also limit our ability to detect fine-scale relationships.

Overall, the results of the geostatistical analyses demonstrate that physical-chemical factors can vary substantially at small spatial scales in soils and within an area that would be classified as a single habitat. These results are particularly striking because the effect of most agricultural practices is to homogenize soils and thus remove variation from nearby sites (Robertson et al., 1993). Nonetheless, they are consistent with results from several earlier studies (Beckett and Webster, 1971; Robertson et al., 1988 & 1993; Webster and Butler, 1976). For example, Robertson et al. (1997) found that > 50 % of the variability in soil properties in a cultivated field resulted from spatial structures between 5 and 60 m. In abandoned fields, Tilman (1982) has shown that levels of important soil nutrients can vary at a scale of meters. Similar patterns have been found in forest soils (Boerner and Koslowsky, 1989; Bringmark, 1989; Bruckner et al., 1999; Palmer, 1990), and even in aquatic systems (Lehman and Scavia, 1982; Smith, 1986), which suggests that variability such as this is likely to exist in most ecosystems.

In this study, the spatial patterns observed for the environmental variables (Figure 8.6) were quite similar to those found in the earlier analysis of community structure (Franklin and Mills, 2003). In particular, the patch size estimates for some of the environmental properties were nearly identical to those calculated for the microbial community properties, and the kriged maps showed similar spatial patterns for the different groups of variables. These similarities could be the result of an active response of the microbial populations to changes in soil microenvironment, or they could be the result of a spurious correlation between the two sets of variables, induced by a common spatial gradient. These two possibilities were explicitly examined using causal modeling

with Mantel and partial Mantel tests; comparisons were made between sets of similarity/dissimilarity matrices to determine whether changes in the microbiological properties were correlated with changes in the environmental variables while considering the spatial separation of sampling locations.

In the first set of analyses, overall community structure was considered using a similarity matrix derived from the AFLP fingerprinting data. The variability in the AFLP patterns was strongly spatially structured at all of the analytical scales considered (Figure 8.7), but did not correlate with environmental variability for any of the physical-chemical properties we measured (after the shared correlation with space was removed (Table 8.4)). However, the models developed for each community subset (PC) showed a strong relationship between changes in community structure and changes in both spatial separation and environmental variability (Figure 8.8). Overall, CN seemed to be the most important environmental factor, as it was included in all of the community subset models. At each analytical scale, community subset PC 2 was also influenced by changes in OM, and changes in community subset PC 3 were correlated with changes in soil texture.

The importance of spatial structure in the causal models decreased at smaller analytical scales. For example, the plot and large-scale models for community subset PC 2 indicate that the distribution of the microbial community is partially caused by changes in CN and OM content, and partially by other factors not explicitly identified but summarized under the term “space”. Moreover, space was also correlated with changes in soil CN and OM, which presents to an additional indirect correlation between space and community structure PC 2. This indirect correlation, via the environmental variables, was also observed at the small scale. At the fine scale, space was not found to be an

important factor for any of the comparisons we made. Similar results were obtained for community subset PC 3. In general, the models developed for the larger spatial scales were more complex and better supported by the data, compared to the models for smaller spatial scales, and this is likely due to the same factors discussed above for the fine-scale variogram analysis. It may also reflect increasing ecological complexity, which is expected for larger spatial scales, in that more variables are interacting to structure the environment.

These models were developed based on how differences in one variable correlated with differences in another variable, but they do not provide any information about the environmental regime associated with each community. To investigate this, a PCA was performed on the environmental data collected from each of the nodes, and a plot of the first two PCs was produced and compared to PC plots created for the community structure data (Figure 8.10). Combined, the first two PCs explained ~ 75% of the variance associated with the environmental properties. The variables important for PC 1 were: sand (factor loading = 0.96), silt (- 0.91), C (0.86), and clay (- 0.80), and the variables important for PC 2 were: OM (0.86). Soils from nodes A and D have high carbon and sand content, and low amounts of silt and clay, while soils from nodes B, C, and X have lower carbon and sand content, and relatively high amounts of silt and clay. In general, the results for the PCA of the environmental variables are quite similar to the results obtained from the AFLP and T-RFLP data (Figure 8.10).

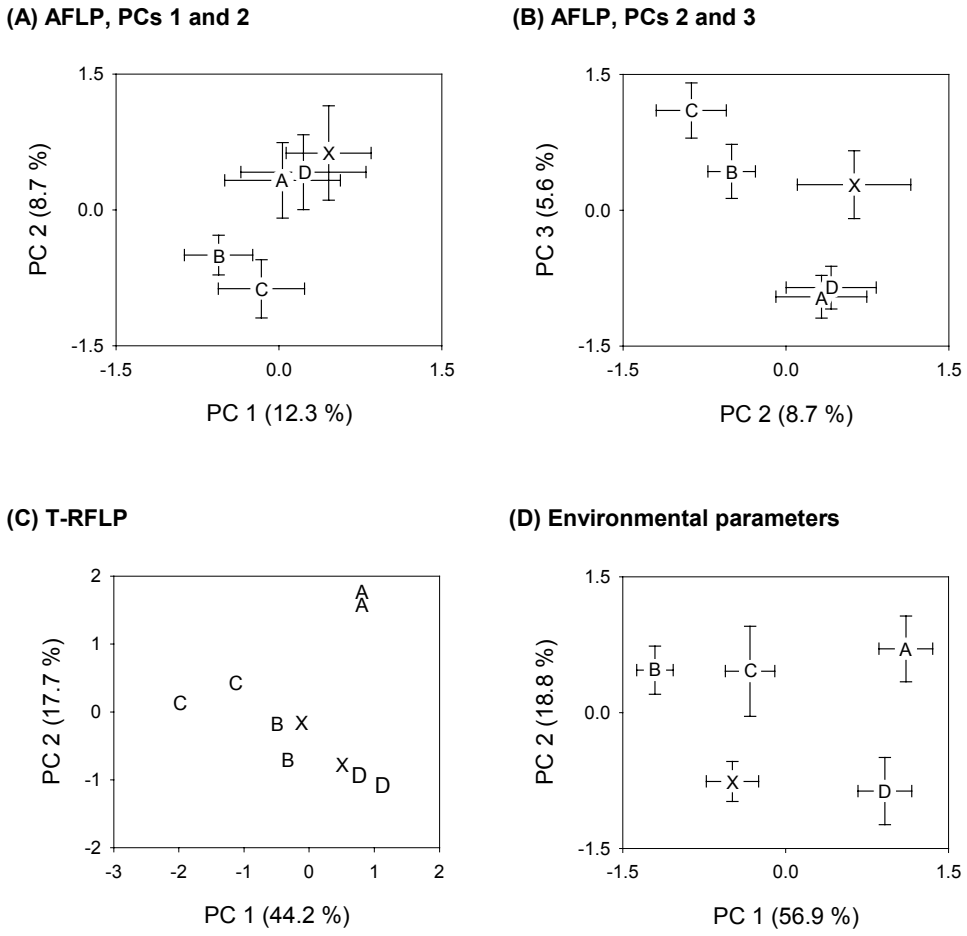

Figure 8.10. Principal components plots. (A and B) AFLP data, (C) T-RFLP data, and (D) environmental data. Error bars represent 99% confidence intervals.

It is not necessarily surprising to discover that patterns in soil C, N, and OM were strong controls on microbial community structure. It is generally accepted that microbial community composition is partially controlled by the amount and type of substrate available, and carbon is often a limiting factor for microbial growth in soil (Aldén et al., 2001). Previous work has also demonstrated the existence of "hot spots" (zones of intense microbial activity and large microbial populations) in relation to available organic matter (Gonod et al., 2003); in this study, a strong relationship was observed between bacterial abundance and soil organic matter (Figure 8.9)

The response of the microbial community to the distribution patterns for C, N, and OM indirectly suggests a relationship between the microorganisms and the distribution of vegetation in the plot. The structure and functional diversity of microbial communities in soils has been show to be tightly related to plant species composition and distribution (Grayston et al., 2001; Kourtev et al., 2003), and there is evidence that certain components of microbial community structure can vary at spatial scales consistent with the distribution of individual plants (Cavigelli et al., 1995; Robertson et al., 1997). It is likely that differences in the age or health of the individual plants can also alter microbial community structure. These changes in the microbial community may, in turn, influence plant growth. For example, changes in the efficiency with which microbial communities decompose organic matter and/or changes in the size or composition of the microbial community have been demonstrated to cause changes in nutrient cycling (Boerner and Rebbeck, 1995; Cotrufo et al., 1994) and in the structure of the plant community (Lambers, 1993).

Compared to C, N and OM, much less is known about the way the physical soil matrix affects microbial community structure and function. Several studies have confirmed that particle size may influence microbial communities, however the nature of the effect seems variable; some reports indicate that reducing particle size results in increased rates of microbial processes (Amato and Ladd, 1988; Bending and Turner, 1999), while others have found the reverse effect (Jensen, 1994; Sorensen et al., 1996). Soil texture has the potential to affect the accessibility of substrate to soil organisms as well as influence many aspects of the soil microenvironment (e.g., the exchange of water, nutrients, and oxygen). Soil texture is also thought to influence microbial community structure by affecting biological interactions between organisms such as competition and predation (e.g., by providing physical protection to prey species (Wright et al., 1995)).

In this study, the causal models were presented with texture "influencing" the microbial community, but not vice versa. This is not entirely accurate, as it has been shown that soil microorganisms may act as 'glue" and mesh soil particles together via exudates and fungal hyphae (Ramakrishnan et al., 2000); this produces clusters of soil that may have properties that are quite different compared to soil not affected by biota (Kristensen et al., 2003; Preston et al., 1999). Our analysis of particle size distribution included chemical treatment and agitation to disrupt this binding, and therefore did not reflect any of the changes in soil texture/aggregation that may have been induced by microbial activity.

There are a number of soil environmental factors that may influence microbial community properties besides those we measured. For example, evidence shows a close relationship of soil microbial communities and pH, soil moisture, and soil temperature

(Emmerling et al., 2001). There are also a number of biotic factors that may be important, including small-scale relationships among the microorganisms (e.g., competition or predation). Furthermore, cm- to meter-scale patterns of spatial variability have also been observed for soil fungi and nematodes, and have been linked with microbial patterns (Ettema and Yeates, 2003; Gorres et al., 1998; Kuperman et al., 1998; Mottonen et al., 1999; Robertson and Freckman, 1995). These relationships are in addition to those associated with plants (discussed above).

It has been suggested that conclusions about the organization of microbial communities, the effects of disturbance, or the roles of various limiting factors are likely to differ at different spatial scales (Wiens et al., 1986). Paradoxes may arise when different investigators, studying similar communities but at different scales, arrive at different conclusions about the factors that structure these communities; these disagreements may reflect viewpoints of different scales, and not necessarily differences in the way communities are organized (Rahel, 1990). The results of this study indicate that one's conclusions regarding the factors that are important for controlling community structure in this system can indeed change depending on the analytical scale used (even within the range of cm to 10 m), but can also greatly depend on the portion of the community studied. In particular, the different 'community subsets' were found to correlate with different environmental properties. This finding has important implications considering that the different techniques available to characterize microbial community structure all have limitations, and are generally biased and focus on particular portions of the microbial community. For example, traditional microbiological methods

are selective for microorganisms that are capable of growing on culture media, community level physiological profiling (CLPP) may be biased toward faster growing organisms (Konopka et al., 1998), and the numerous molecular genetic methods may provide very different results depending on differences in nucleic acid extraction procedures, PCR conditions and primers, or the resolution associated with a particular technique. The results of our study suggest that researchers must be especially careful about comparing separate community-environment studies that use different assays to evaluate community structure; the findings may change considerably depending on the portion of the community actually evaluated.

In this study, we used two very different DNA fingerprinting techniques (AFLP and T-RFLP), with different levels of resolution, to characterize microbial community structure. T-RFLP is a technique that can be used to examine differences between microbial communities based on variability in the 16S ribosomal RNA gene. It is particularly useful when applied in conjunction with clone libraries and DNA sequencing, as it allows for the identification of community members. However, it is well known that T-RFLP underestimates the species richness of a community because populations that are not numerically dominant are not represented if their template DNA constitutes too small of a fraction of the total community DNA pool (Dunbar et al., 2000; Liu et al., 1997). Moreover, because T-RFLP is insensitive to changes in community composition that may occur at the level of individual strains or species (Buckley and Schmidt, 2001), microbial communities whose overall structure appears similar by T-RFLP analysis may still possess ecologically significant differences in community composition.

AFLP is a DNA fingerprinting technique in which a restriction digest is performed on a DNA sample (similar to RFLP), and then a set of primer-recognition sequences (adaptors) is used to amplify the restriction fragments using PCR (Zabeau and Vos, 1993). The primers and restriction enzymes used are not specific for a given gene or group or genes but can, theoretically, interact in numerous random places throughout a genome, making AFLP a useful technique for analysis of *overall* differences between communities, including strain- or species-level changes. Variability at this taxonomic level is still ecologically important and may be responsible for differences in the physiological capacity of different microbial communities. One major limitation of AFLP is that lack of a relationship between characters in the DNA fingerprints (e.g., the size of a band) and any species or genus identification. Consequently, AFLP profiles provide a means of determining differences between communities', but fail to provide insight into the identity of the specific organisms responsible for those differences. In our study, this means that we cannot identify the organisms responsible for the differences between our community subsets (PCs), but there are a number of other techniques that could be applied to our DNA samples if that information was of specific interest.

Spatial variability, such as we observed in this study, is likely to exist in most ecosystems, and needs to be considered when making inferences about ecological relationships and when developing strategies to sample the environment (Robertson et al., 1997). In particular, understanding the scale at which a parameter must be measured is essential to creating a sampling design that will result in a sound ecological evaluation of that parameter, and in determining sample sizes and appropriate statistical techniques for

data analysis. However, the extent to which the specific findings presented in this study can be extrapolated to other times and sampling scales at this site, or to different sites, is unclear. Furthermore, this study only considered horizontal variability across the field, and did not investigate changes associated with vertical depth into the soil. Most studies in soil microbiology have focused exclusively on the surface layers of the soil, and less is known about the nature of microbial communities found throughout the soil profile (Fierer et al., 2003). Deeper layers of soil may contain microbial communities that are specialized for their environment and fundamentally different from the surface communities, and recent studies have found that the composition and structure of soil microbial communities changed significantly with soil depth (Blume et al., 2002; Fierer et al., 2003; Griffiths et al., 2003; Zyagintse, 1994).

The results presented in this paper provide a snapshot of the relationships in this field at a single time, and do not consider the role of temporal variability, or its interaction with spatial heterogeneity, in determining community patterns. Because different environmental variables are important not only at different spatial scales, but also at different temporal scales, studies that examine both simultaneously are needed. Some of the soil properties we measured are expected to be relatively static (e.g., sand or silt content), while other, such as C, N, or OM are expected to be more seasonally dynamic. Still others, which we did not measure, such as temperature or water content, may cause variations over even shorter time scales. Changes in microbial communities have also been shown to arise due to tillage practices, input of fertilizers, organic residues, and pesticides, and crop rotations. For example, it has been reported that

microbial biomass can fluctuate sharply over days following agricultural management or natural influences like drying and rewetting of a soil (Ocio et al., 1991 a & b; Wyland et al., 1995).

While increased knowledge of these many factors influencing microbial community structure, and the role of space and time in this relationship, is important, there is particular interest in understanding how these factors may affect the activity of microorganisms in an ecosystem. Soil microbes play a crucial role in keeping the main nutrients cycling in soils (C, N, P, S), and are fundamental for the long-term functioning of ecosystems. The results from the present study demonstrate that changes in community structure may occur is association with different environmental conditions; however, it remains to be determined how/if this change in structure will manifest as a change in microbial community function. Little is known of the importance of microbial community structure and diversity in the functioning of soils (Degens, 1998; Giller et al., 1997; Pankhurst et al., 1996), though it is often hypothesized that diversity is important for the maintenance of soil processes, and that reductions in soil microbial diversity will disrupt the functional capability of soils (Giller et al., 1997). However, considering the incredible diversity of microbial systems, the extreme physiological versatility of microorganisms, and the high level of functional redundancy thought to be present in microbial communities, this may not be the case. The few studies available that specifically address this hypothesis in soils present conflicting results (Atlas et al., 1991; Klein et al., 1986; Salonius, 1981), though there is some evidence to suggest a link between the microbial community *structure* and function. For example, Zogg et al. (1997) studied the structure of microbial communities using phospholipid fatty acid

profiling (PLFA) and function using respiration kinetics, and concluded that changes in the communities' structure during soil warming were related to changes in function. Similarly, PLFA profiles have been correlated with enzyme activities in several different soil systems (Kourtev et al., 2003; Waldrop et al., 2000). Nevertheless, at this point, it is not possible to extrapolate information about microbial community function from an analysis of the structure of the community (Degens, 1999). Increased research into the relationship between structure and function is necessary before scientists can anticipate how habitat disruption and changes in community structure may impact community activity and ecosystem performance, and a better understanding of the role of spatial heterogeneity in microbial communities with help ecologists to determine the relevance of small-scale observations and experiments for large-scale patterns and processes.

Chapter 9.

Conclusions and synthesis

9. Conclusions and synthesis

The results presented in this dissertation demonstrate the importance of research into issues associated with spatial scale and microbial communities. The work provides much needed experimental and statistical information on spatial variability of microbial communities and the relationship of community organization to environmental heterogeneity. The important results and conclusions may be divided in three general (but not exclusive) categories: (i) methods for microbial community analysis, (ii) conclusions regarding spatial structure in microbial systems, including issues related to statistical techniques and experimental design, and (iii) the relationship of microbial community structure and function. The major results for each topic are presented below. A discussion of how these results relate to other recently published work is included, as are recommendations for future research.

9.1. Conclusions regarding methods for microbial community analysis

9.1.1. Arbitrarily primed PCR-based DNA fingerprinting techniques such as RAPD and AFLP are useful means of analyzing microbial community structure.

The work presented in this dissertation demonstrates that DNA fingerprinting techniques such as these are a useful means of discriminating among microbial communities and estimating relative community similarity. In general, the results obtained using RAPD and AFLP were quite reproducible and consistent with other techniques for whole-community analysis of microbial assemblages. Results obtained using RAPD and AFLP were compared with many commonly used whole-community

approaches including DNA-DNA hybridization (Lowit et al., 1998), community-level physiological profiling (CLPP), and molecular genetic techniques based on analysis of the 16S rRNA gene. The results of these comparisons are discussed in more detail in Sections 9.1.2 and 9.1.3.

RAPD and AFLP are fairly easy to perform and may be readily adapted for use with many different types of samples. As part of my dissertation work, I used these procedures to analyze microbial communities isolated from tidal creeks, aerobic and anaerobic groundwater, salt marsh sediments, sewage, and agricultural soil. One particular advantage of the RAPD approach is that it does not require as much specific equipment as most other molecular genetic techniques, and thus may be employed in a laboratory that does not necessarily specialize in such analyses. More specifically, RAPD does not require that a researcher have access to a DNA sequencer or capillary electrophoresis equipment, as does T-RFLP. Moreover, useful RAPD profiles may be obtained using simple agarose gel electrophoresis and staining with Ethidium Bromide, whereas procedures such as DGGE or AFLP demand the use of more complicated electrophoresis equipment and/or alternate staining procedures.

9.1.2. Genotypically-based whole-community analyses provide similar results to phenotypically-based analyses of community structure.

Throughout my dissertation research, several different molecular genetic techniques (RAPD, AFLP, DGGE, and T-RFLP) were compared to techniques based on the differential physiological response of different microbial communities (traditional CLPP and dilution-to-extinction CLPP, as well as traditional plate counts). Similar

results were obtained using each technique, but the procedures differed in their resolution and in the specific type of information they provided. Moreover, different techniques were sensitive to different types of changes in community structure (e.g., changes in the distribution of types versus changes in the identity of types). The different, but complementary, results from the various procedures highlights the importance of using multiple techniques to evaluate microbial communities in ecological studies.

Integrated diversity studies, based on the combined use of molecular methods, physiological measurements, and appropriate culture-based analyses, have been recommended by a number of researchers (Chandler et al., 1997; Grundmann and Gourbiere, 1999; Head et al., 1998), but are not commonly performed. In the present work, the conclusions drawn from the use of any individual analytical technique were not as valuable as the interpretation of these conclusions in the context of the results from the other techniques. Moreover, it is important to point out that the "restrictions" of a particular technique are not necessarily always "limitations", and can be manipulated to the advantage of a researcher when specific questions are of interest. For example, the T-RFLP technique was limited in that it only provided information regarding the distribution of dominant organism types (Torsvik et al., 2002); however, in some situations that type of information is particularly useful. In the research presented here, the information was particularly valuable when comparing the results from the dilution/regrowth experiments to the numerical simulations (Chapter 3 and 4).

The results presented in this dissertation also allow for a comparison of genotypic and phenotypic approaches to microbial community analysis. The fact that these two sets of methods provide similar results demonstrates that differences in genotype actually

have the potential to manifest as differences in the function of microbial communities. Often times, when researchers are advocating the use of molecular methods over culture-based procedures and CLPP, the reason presented is that the later procedures are too biased toward certain groups of organisms. In particular, it has been suggested that CLPP results reflect changes in community structure that are primarily due to differences among fast growing copiotrophic organisms (Konopka et al., 1998), and traditional culture-based techniques are strongly influenced by the environmental conditions and growth substrate used in the assay. The results presented in this dissertation demonstrate that these limitations are not necessarily any more restrictive than those associated with the various molecular analyses. Moreover, the role of classical microbiology should not be undervalued as new and more advanced molecular genetic procedures are developed. These genetic analyses permit cataloging of organisms based solely on DNA sequences, thus permitting assessments of diversity and community structure, but provide no information on the functional role an organism plays (or is capable of playing) in an ecosystem.

9.1.3. Analyses of microbial community structure that rely upon examination of 16S rRNA genes may not be as accurate or as functionally relevant as techniques that consider variability throughout the microbial genome.

With T-RFLP, PCR is used to amplify the 16S rRNA genes directly from a sample of community DNA, and analysis produces a fingerprint wherein each individual band (T-RF) is, theoretically, derived from a different type of organism (Liu et al., 1997). However, it is well known that T-RFLP underestimates the richness of a community because populations that are not numerically dominant are not represented if their

template DNA constitutes too small a fraction of the total community DNA (Dunbar et al., 2000; Liu et al., 1997). Moreover, due to the conservation of restriction site positions in the 16S rRNA gene, the resolution of T-RFLP is not at the species level (Buckley and Schmidt, 2001), but instead reflects the distribution of higher order groups.

With AFLP, a restriction digest is performed on a DNA sample (similar to RFLP), and then a set of primer-recognition sequences (adaptors) is used to amplify the restriction fragments using PCR (Zabeau and Vos, 1993). In contrast to T-RFLP, the primers and restriction enzymes used are not specific for a given gene or group or genes, but can interact in numerous random places through a genome. AFLP is fundamentally different from most of the other techniques used to analyze microbial community structure in that it is sensitive to overall differences between communities – including taxonomic distances between community members.

In my comparisons of T-RFLP and AFLP, whenever significant changes in community structure were observed using T-RFLP, the same results were obtained with AFLP (Chapters 3, 4, and 8). However, AFLP consistently revealed additional significant differences that were not detected with T-RFLP. For example, in the dilution/regrowth experiments discussed in Chapter 4, T-RFLP separated the various dilution/diversity communities in two distinct groups, while AFLP divided the communities into three significantly different sets.

The results of the comparison of these two techniques confirm that the AFLP procedure is not limited to the detection of dominant organism types to the same extent as T-RFLP. The identification of a technique that responds to changes due to rare(r) species is particularly important, given that so many of the methods currently used focus on the

characterization of the dominant organism types. Those dominant organisms may actually represent a small portion of the total microbial community; for example, in water only about 10 % of the simultaneously coexisting species are dominant (Torsvik et al., 2002). The development of techniques that better consider the contribution of rare organism types to overall community structure and function is an important area for further study. Alternately, it is possible that the difference between the two methods is due to the fact that AFLP considers variability throughout the microbial genome, and not just that associated with a single gene. This could make the technique more sensitive to overall differences between communities, including strain- or species-level changes.

Additional analyses were performed to examine the relationship of AFLP fingerprinting and T-RFLP to overall community function (Chapter 4). The fact that there were no significant correlations between T-RFLP community structure and community function, but strong correlations for AFLP, suggest that the analysis of overall community structure using AFLP may be a better predictor of potential/actual changes in community function, compared to an analysis of the 16S rRNA gene (via T-RFLP). Given that metabolic functions are seldom phylogenetically grouped (Ward et al., 1995), this is not necessarily surprising. Because AFLP fingerprints reflect variability present throughout the entire community DNA pool, differences in functionally relevant genes can also contribute to the AFLP profile, and thus may explain the correlation observed between the AFLP community structure assay and community function. These results indicate that microbial communities whose overall structure appears similar by T-RFLP analysis may still possess ecologically significant differences in community composition. An alternate explanation for the results presented here is that the

correlation between AFLP-based community structure measurements and activity is partially due to the inclusion of rarer organisms in the structural assay. Overall, the results of the comparison of these two techniques suggests that the AFLP procedure may be a more useful technique for analysis of overall differences between communities and may be a better predictor of potential/realized differences in community function.

9.1.4. The dilution/regrowth approach is a useful method for studying microbial community structure.

The dilution/regrowth approach has been used several times as means of establishing microbial communities differing in structure and relative diversity (Garland and Lehman, 1999; Garland et al., 1999; Griffiths et al., 2001; Mills et al., 2003; Morales et al., 1996; Salonius, 1981). In general, the premise behind each of these studies was that dilution of a complex community will exclude organism types, creating mixtures of cells differing in diversity. However, the actual effect of dilution on diversity has not previously been quantified. The numerical simulations presented in Chapter 3 demonstrate that, though the basic premise behind the dilution/regrowth approach is valid, its application to diverse microbial communities requires careful consideration of the initial distribution of the community. The results also indicate that the dilution/regrowth approach is itself a useful means of analyzing a microbial community; a great deal of information about the richness and distribution of the original community may be gained by analysis of the regrown assemblages (Chapters 3 and 4).

Overall, the results of the dilution/regrowth studies indicate that the approach is a useful means of creating communities differing in community structure and overall diversity for future use in experimental studies. This approach has been used to study the stability and resilience of microbial communities to several types of perturbations (Garland et al., 1999; Mills et al., 2003; Morales et al., 1996), and the work presented in this dissertation provides important information regarding the types of community changes that would have been considered in the previous work. Unfortunately, the dilution/regrowth approach does not provide much flexibility for manipulating community diversity, nor does it allow for any manipulation based on the functional traits of either the individual organism types or the combined community. More comprehensive studies that consider differences in microbial communities beyond that which is possible using dilution/regrowth are needed. In order to more fully examine the role of properties such as community composition and structural diversity, functional diversity, or functional characteristics of specific organism types, it will be necessary to work with constructed communities, so different aspects of the community may be more fully manipulated and more careful controlled.

Most studies using constructed communities do not work with mixtures that are very similar to natural communities; that is, they frequently comprise too few types and use combinations of organisms that may not occur in natural environments. Recent work by Button et al. (1993) describes an “extinction culturing” approach that may be useful for obtaining large numbers of isolates from individual communities, which could then be used for more comprehensive studies with constructed communities that have a higher richness and use naturally co-existing types. In its original form, the extinction-culturing

approach was very laborious; however, high-throughput methods have recently been developed to enable isolation of a large number of types of microorganisms relatively quickly (Connon and Giovannoni, 2002). Using communities constructed with this approach would allow us to manipulate different aspects of diversity, and then examine the relationship of diversity to other community-level properties such as productivity and stability.

9.2. Conclusions regarding spatial structure in microbial systems, including issues related to statistical techniques and experimental design

9.2.1 The application of geostatistical techniques to the analysis of community fingerprinting data can provide useful information regarding the spatial organization of microbial communities.

Geostatistics is a set of statistical tools for incorporating the spatial coordinates of sampling observations into data processing. These tools can provide a powerful means of quantitatively describing spatial variation by expressing a measure of association, or autocorrelation, between two samples as a function of the distance between them. When applied to microbiological data, most of the analyses have focused on either the μm to cm-scale distribution of individuals (Dandurand et al., 1995 and 1997; Grundmann and Debouzie, 2000; Nunan et al., 2001 and 2003), or the larger-scale distribution of total microbial biomass and abundance (Moran et al., 1987; Mottonen et al., 1999; Robertson et al., 1997; Saetre, 1999; Smith et al., 1994; Troussellier et al., 1993); the techniques are rarely used to analyze community composition or microbial diversity (Cavigelli et al., 1995; Mackas, 1984; Saetre and Bååth, 2000).

One of the important contributions of this dissertation research was the application of geostatistical methods to the analysis of spatial patterns in microbial community structure, particularly to the analysis of DNA fingerprinting data. The number of studies in microbial ecology that use molecular techniques is growing rapidly, and this rate can be expected to continue to increase with the further improvement of methods particularly tuned for environmental work. However, progress in developing or applying quantitative statistical techniques to the analysis of these data has been much slower. This is true not only in the field of spatial analysis, but throughout environmental microbiology. For example, much of the information that is available regarding changes in community structure in response to environmental perturbations relies upon the visual interpretation of DNA fingerprinting patterns, but what is needed is a means of quantifying these differences and determining their significance in order to make meaningful comparisons between different experiments and laboratories. DNA fingerprinting data are particularly amenable to multivariate methods and other statistical procedures that are typically used to examine taxonomic diversity or community composition for macroorganisms, and these techniques may often be applied to the analysis of microbial communities with relatively little modification. For example, principal components analysis, cluster analysis, and Mantel tests have been used to analyze DNA fingerprinting data to address these types of questions. The ability to quantitatively compare microbial communities is an important step toward more sensitive monitoring, and eventually, being able to integrate data gathered by different researchers so that overall patterns may be identified. It is important that microbial ecologists

become more aware of the availability of these techniques, and consider their use when analyzing and interpreting microbial community data.

Because the DNA fingerprinting data collected as part of this dissertation research were multivariate and binary (presence or absence of bands), the direct application of traditional geostatistical variogram analysis was not possible. However, by converting the DNA fingerprinting data into similarity and dissimilarity matrixes, and then applying a variogram analysis to these matrices, a great deal of information on the spatial patterns of these assemblages was obtained. Using this pseudo-variogram approach, it was possible to determine the correlation length scales ("range") of the microbial community and the spatial dependence (the percent of variance in the data that may be due to spatial autocorrelation structure). A great deal of spatial autocorrelation in microbial community structure was detected, and different microbial community attributes (e.g., abundance versus community structure) displayed different spatial patterns (Chapters 6 and 7). Changes in these different community attributes were correlated with changes in different environmental variables, which had different spatial patterns (Chapter 8). This demonstrates that the analysis of a single community property is not sufficient to determine the important environmental variables controlling the development and organization of a microbial assemblage.

The work presented here also demonstrated that a great deal of the variance observed in microbial community structure at the cm to 10-m scale may be due to spatial autocorrelation; the values obtained indicate that between 9 and 73 % of the variance could be explained by considering the spatial separation of sampling locations. Understanding the amount of variance in a dataset that is due to spatial autocorrelation

may be particularly important is situations where one is trying to develop models to explain the relationship of microbial community structure, diversity, or function, to fluctuations in various environmental properties. For example, one may develop a model using three environmental variables, and discover that only 50 % of the variance in community structure is explained. By determining the amount of variance that is due to spatial autocorrelation, a researcher can better decide the value of examining more environmental properties and searching for other causes of community variability. If a large portion of the remaining variance may be accounted for by considering the spatial separation of sampling locations, then it may not be necessary to consider other physical or chemical properties that could be influencing community structure. However, if little spatial structure is detected, further investigation of additional environmental variables may be appropriate. Another advantage to including data on spatial structure in ecological analyses and models such as these is that much better predictions may be obtained when the spatial structure is included among the predictive variables (Legendre, 1993).

The results of a geostatistical analysis may provide the basis for interpolation by kriging, and therefore allow for the production of maps displaying the spatial distribution of community structure at sampled and unsampled locations. These techniques are particularly valuable because the methods may be applied to environmental data, and the distribution of the community may be compared with the physical-chemical habitat variation at a site (Chapter 8). The same approach may be used to generate maps of community function (e.g., uptake of a particular substrate), which would be particularly valuable for predicting/calculating overall community activity in an area.

9.2.2. Significant spatial autocorrelation exists in microbial community structure, even in environments that may seem "homogeneous". This structure can manifest at many different nested scales, and may influence analysis of microbial community patterns.

The presence of spatial autocorrelation in microbial systems has been demonstrated by a number of researchers, as outlined in the Chapter 1 of this document. The results presented in this dissertation are consistent with this earlier work, and help to confirm the notion that spatial autocorrelation is a generic feature of ecosystems that needs to be considered when examining patterns of microbial community structure and function. Even in the agricultural field (predicted to be the most homogeneous site), a great deal of multi-scale spatial autocorrelation was detected. The fact that spatial autocorrelation was so widespread, across such a range of scales and in so many different types of environments, further supports the claim that spatial variability needs to be considered when making inferences about ecological relationships and when developing strategies to sample the environment (Robertson et al., 1997). The identification of such patterns can influence the selection of statistical techniques used for data analysis, and failure to compensate for autocorrelation can lead to incorrect statistical conclusions. Moreover, the results of the present research indicate that one's conclusions regarding the factors that are important for controlling community structure in a system can indeed change depending upon the analytical scale considered (even within the range of cm to 10 m).

The importance of spatial structure in experimental design and statistical analysis is frequently commented on, but rarely examined. Despite increasing recognition of the importance of spatial autocorrelation in environmental samples, most microbial

ecologists still do not consider this aspect when designing a sampling scheme. A review of more than 50 papers published in major journals between 1993 and 1995 showed that when researchers studying microbial properties in the field actually specified their sampling design (which was only 63% of the papers), a random sampling pattern or random transect was used 96% of the time (Morris and Boerner, 1999). However, if microbial properties are as strongly influenced by small-scale environmental variability as is currently suspected, then the habitat is not structured randomly, and the use of random transects may not be appropriate (Legendre et al., 1989).

Some procedures exist that allow researchers to make corrections and perform statistical analyses in the presence of spatial autocorrelation – but these corrections are often difficult to perform, and their efficacy is uncertain (for an overview, see Dutilleul and PinelAlloul (1996), Legendre and Legendre (1998), and Legendre et al. (1990)). In some cases, it may be possible to remove large-scale spatial structures from a dataset by regression or model-fitting, in order to carry out classical statistical analyses on the residuals. However, in doing so, one must be careful not to remove one of the important determinants of the processes under study, since spatial heterogeneity may be functional in ecosystems (Legendre and Fortin, 1989). Another solution to deal with natural spatial autocorrelation is to design a sample collection scheme so that there is little spatial structure present in the data, and then use parametric statistical hypothesis tests. In this case, samples must be collected close enough together that they represent replicates of the system under investigation, but they must be far enough apart to avoid autocorrelation. This approach requires prior knowledge about the spatial patterning of the variables, obtained from previous surveys or a pilot study.

Regardless of which approach one chooses (correction of statistical procedures or modification of experimental design), it is first necessary to describe the type of autocorrelation present in a system (e.g., gradients versus patches) and then estimate its extent. However, because it is not always feasible to first do an extensive reconnaissance survey, and because the results presented in this dissertation suggest that one is not likely to avoid the impact of spatial autocorrelation, a more reasonable solution may be to include spatial separation as a part of routine data collection. An initial analysis of this information can then be used to determine the influence of spatial autocorrelation on the dataset. If significant spatial structure is found, this information must be considered as a variable and incorporated into subsequent data analysis; if not, traditional parametric techniques may be appropriate.

9.2.3. Different portions/subsets of the microbial community may be spatially distributed in different ways, and in response to different environmental variables.

Significant spatial autocorrelation in microbial community structure was observed for both the salt-marsh creek bank sediments and the agricultural wheat field (Chapters 6 and 7). Moreover, the results of the wheat field study demonstrated that different subsets of the microbial community were distributed in different ways, and this was found to be due to the differential response of these community subsets to various environmental properties (Chapter 8). In particular, three community subsets were examined. All three groups were influenced by changes in soil CN availability, one group was also influenced by changes in OM concentration, and another group was influenced by changes in soil

texture (as well as CN). As a result, the spatial distribution and autocorrelation structure associated with each community subset was distinct.

These data imply that different groups/types of organisms may respond differently to environmental stress and perturbation; therefore, to build a true picture of the impact of these disruptions, it may be necessary to examine several components of the community (and ecosystem). These results also demonstrate that researchers must be especially careful when comparing separate community-environment studies that use different assays to evaluate community structure, as the findings may change considerably depending on the portion of the community actually evaluated. This is particularly important, considering that the different techniques available to characterize microbial community structure are each biased toward particular portions of the microbial community. For example, traditional microbiological methods are selective for microorganisms that are capable of growing on culture media, community-level physiological profiling (CLPP) may be biased toward faster growing organisms, and the numerous molecular genetic methods may provide very different results depending on changes in nucleic acid extraction procedures, PCR conditions and primers, or the resolution associated with a particular technique. Analyses using these different techniques may be expected to provide similar *overall* results (e.g., separation of distinct communities using PCA, as discussed in Section 9.1.2), but may not be as well matched when analyzed in more detail using geostatistical techniques. Therefore, studies that use statistical techniques to compare spatial variability, community structure, and environmental variability are more likely to generate different results when different methods are used to characterize community structure. Further research that focuses on

creating multiple maps of the community structure in an area, using different laboratory methods to analyze the communities, would prove some information of the magnitude of this discontinuity.

9.2.4. Sample size may be a more important consideration when analyzing microbial community structure, compared to overall community function.

The relative scales between what constitutes the size of a habitat required for a prokaryote, and the size of samples typically taken for observation in environmental studies, is an important consideration that has motivated much of the research presented in this dissertation. Though this is a very important element of spatial analysis and the consideration of spatial relationships in microbial communities, experiments addressing the issue of sample size did not constitute a large portion of this dissertation research. Nonetheless, some conclusions may be drawn from the dilution/regrowth experiments, and a general discussion of this issue is appropriate.

The results of the laboratory studies indicate that the sample size used for community analysis may be a very important consideration when examining overall community structure, but is less of an issue when comparing community function. Moreover, differences in microbial community function could not be inferred from observed differences in community structure, and this is likely due to the widespread functional redundancy present in microbial systems (to be discussed in Section 9.3). The impact of sample size on the perception of microbial community structure and activity depended on the relative distribution of the organism types within the meta-community. If the community has a relatively even distribution (approximately equal numbers of each

type), then changes in sample size will not necessarily lead to changes in the perceived community structure – until the sample size is so small that the number of organisms collected is less than the richness present in the largest sample size. This result was not particularly surprising, and implies that differences in community structure will be only detected when samples sizes differ by several orders of magnitude. However, the results indicate that very large differences in community structure may be observed over relatively small intervals (e.g., 0.1 ml to 1 ml) if the community is not evenly distributed.

Other researchers have also determined that changes in sample size may influence the analysis of microbial community structure (Ellingsoe and Johnsen, 2002; Kirchman et al., 2001; Long and Azam, 2001) and abundance (Duarte and Vaqué, 1992; Mitchell and Fuhrman, 1989; Muller-Niklas et al., 1996; Seymour et al., 2000). For example, in forest soils, Ellingsoe and Johnson (2002) observed a great deal of variation in genetic community structure when comparing DGGE fingerprints created from replicate 0.01 g samples. Variation was also observed between 0.1 and 1.0 g samples, whereas variation between 1.0 and 10.0 g samples was negligible. Work examining variation in community function in association with changes in sample size has also been performed, but to a lesser extent (Parkin et al., 1987). The results presented in Chapter 4 are the first where the influence of sample size on both community structure and activity has been considered. The results demonstrate that sample size and spatial heterogeneity are important factors, as other researchers have determined, but that community composition (specifically, the distribution of types) and the level of functional redundancy present in the system may also influence the results of such an analysis

One important factor controlling the sample size used in microbiological analyses is the limitations imposed by analytical requirements. However, advances in molecular genetics, image analysis, and micromanipulators make this less of an issue. For example, Kirchman et al. (2001) achieved positive PCR with as little as 25 μl of coastal water. Theoretical calculations suggest that even smaller samples may be used (Franklin et al., 1999). Despite a recent increase in research associated with the issue of sample size and understanding how our perception of the composition and distribution of a microbial community changes when different spatial extents are considered, there is still no consensus as to what factors are most important for determining the appropriate sample size for a particular environment or set of ecological questions.

One point that is so fundament to the topic of microbial communities that it is often overlooked is the question of how to define the limits of a microbial community. Most often, the boundaries used to define a 'community' are utilitarian and dictated by the required sample size and the researcher's perception of environmental variability. The size of the sample is crucial, because it determines the number of bacteria that will be subjected to the same sample processing. Generally, the "mere fact that organisms co-exist at the moment of sampling is taken as evidence that they are part of a community… (while) the 'community' may be no more than a disparate collection of organisms that happen to found within a sample of a particular size" (Harris, 1994).

In the environment, it is difficult to delimit microbial communities (impossible in most situations), and separate the cells and species that are members of a community from those that are not. Functionally defined microbial communities exist in continuity with one another, and the distinctions between them blur. These "communities" are

functionally connected by fluxes of organisms, materials, energy, and information. The localized activity of these individual "unit" communities combines in a mosaic pattern to mediate processes that are important at the field and landscape scale. Moreover, microbial activity at the ecosystem level results from the further combined interaction of these larger-scale communities. One's perspective within this hierarchy can be crucial to understanding the pattern or process of interest, and studies that consider multiple spatial scales when studying environment-community interactions are particularly valuable. It is important to point out that most systems cannot be neatly subdivided into hierarchical scales of organization, and there is no single natural scale on which ecological phenomena should be studied (Levin, 1992). The description of the system will vary with the choice of scales, and, rather than trying to determine the "correct" scale, ecologists must try to understand how the system description changes across scales (Levin, 1992). Moreover, learning to scale up from individual measures of environmental samples to processes at the field and landscape scale requires an understanding of how information is transferred from fine scales to broad scales – this requires that scientists learn to aggregate and simplify, retaining essential information without getting bogged down in unnecessary detail (Levin, 1992).

9.3. Conclusions regarding the relationship of microbial community structure and function

9.3.2. Functional differences in microbial communities cannot be inferred from observed differences in community structure.

There is a considerable lack of knowledge regarding the relationship of microbial community composition and community physiological activity. Although it is frequently

proposed that there is a tight link between the two in microbial systems (Finlay et al., 1997; White, 1995), the research presents conflicting results (Atlas et al., 1991; Eddison and Ollason, 1978; Mills and Mallory, 1987; Salonius, 1981; Wassel and Mills, 1983; Xing et al., 1997). The results included in this dissertation demonstrate that there can be changes in the structure of a microbial community that are not necessarily reflected by changes in the function of the community. More specifically, the dilution/regrowth procedure discussed above was used to create communities that differed in both overall structure and diversity. These communities were incubated in the same environmental conditions, and the *in situ* function of the communities was observed by examining the community heterotrophic uptake for five different ^{14}C labeled compounds. There were no significant differences between treatments in either the rate of uptake of a substrate or the assimilation efficiency for any of the compounds studied. This finding demonstrates that it is not necessarily appropriate to draw conclusions regarding changes in community function from an analysis of community structure, and is particularly important given the extensive use of structural assays in ecological studies of microbial systems.

One of the limitations of this study is that community function was only determined for a small group of substrates. However, the compounds were chosen to represent a range of different types of chemical groups: amino acids, short and long-chained carboxylic acids, and carbohydrates. It is certainly possible that differences in function might have been found if the communities had been presented with more exotic compounds. There is considerable debate among ecologists as to what processes should to be chosen to best characterize ecosystem or community functioning (Ghilarov, 1997; Gitay et al., 1996), and the study reported here only addresses the metabolic uptake of a

small group of compounds. Ideally, a more complete analysis of the function of microbial communities would include measures of other compounds and processes, and would also evaluate community stability (resistance and resilience). There is some evidence that suggests that well-defined microbial functions such as nitrification and methane oxidation, which are carried out by a limited microbial sub-set, may be more sensitive to changes in diversity than broader-scale functions such as respiration or decomposition (Griffiths et al., 2000; Toyota et al., 1999; Wu et al., 2002). Kandeler et al. (1996) also showed that carbon cycling may be less sensitive to changes in microbial community composition than nitrogen and phosphorus dynamics.

There are a number of other studies that have also found that the broad-scale functional ability of a microbial community is often not controlled by organism diversity or community structure (Andrén et al., 1995; Finlay et al., 1997; Griffiths et al., 1997, 2000 and 2001; Mills et al., 2003; Salonius, 1981; Toyota et al., 1999). However, despite the fact that structural assays of microbial communities are not necessarily useful for determining these types of changes in community function, there are still a number of ways in which structural analyses may be useful. In particular, an analysis of community structure may be helpful for predicting changes associated with other aspects of community function (e.g., stability), or could be useful as an indicator of environmental change. For example, Boon et al. (2000) examined DGGE fingerprints of microbial community structure in samples collected from different depths in a sediment landfill, and concluded that DGGE was a more sensitive means of characterizing environmental habitat variability than the standard physical-chemical approach. Statistical analysis of the physical-chemical data revealed no significant differences or clustering patterns

associated with depth into the landfill; however, the molecular methodology uncovered consistent and reproducible differences in the banding patterns associated the less dominant bacterial species in different regions of the landfill.

The results presented in this dissertation also demonstrate that changes in community structure may occur is association with different environmental conditions; however, it remains to be determined how/if this change in structure will manifest as a change in microbial community function. At this point, it is not possible to extrapolate information about microbial community function from an analysis of the structure of a community. However, the fact that there is consistently a link between community structure and environmental variability (i.e., in this study, different environments always have communities with different structures) indicates that there probably is a relationship between structure and function, but it is not something that can be detected or explained at this point. Increased research into the relationship between structure and function is necessary before scientists can anticipate how habitat disruption and changes in community structure may impact community activity and ecosystem performance, and a better understanding of the role of spatial heterogeneity in microbial communities will help ecologists to determine the relevance of small-scale observations and experiments for large-scale patterns and processes. Analysis of these relationships over a variety of spatial scales is important because evidence of diversity-function relationships at local scales cannot necessarily be directly extended to regional scales (Hughes and Petchey, 2001).

9.3.3. Functional redundancy may be widespread and an important factor controlling the stability of microbial communities.

In the dilution/regrowth experiments discussed above, the fact that community function was maintained, despite the loss of diversity and change in community structure, indicates that functional redundancy was quite high within the original microbial consortium. For each organism type eliminated by the dilution procedure, at least one of the remaining organism types was able to provide the same function, at the same level, as the lost type. In general, microbial communities are thought to possess a high degree of functional redundancy, which refers to the situation in which many species within an ecosystem are capable of carrying out the same individual function (Roberts et al., 2003). Several researchers have speculated that this redundancy in function is much more important for understanding the stability of microbial communities, and of the ecosystem functions they perform, than traditional diversity measures (Beare et al., 1995; Finlay et al., 1997; Kennedy and Smith, 1995; White, 1995). The hypothesized relationship between functional redundancy and stability within ecosystems is conceptually similar to a cybernetic control mechanism called "congeneric homotaxis" (Hill and Wiegert, 1980). In this case, stability is conferred on a system because multiple genera are capable of carrying out a given function in the ecosystem, presumably across a wide range of environmental conditions. If one of the organisms is eliminated from the system, or if the organism ceases to function for any reason, another organism present within the system (one that is “ecologically equivalent” (Gitay et al., 1996)) provides the function, allowing maintenance of the system’s functional ability at or near the level prior to the loss of the

first organism. This redundancy is often though of as an insurance again the loss of function from a community.

The results presented in this dissertation indicate that functional redundancy may be widespread among microbial communities, and may be an important factor controlling the functional stability of these assemblages. However, it is important to point out that there are several reasons why *overall* ecosystem functioning may not be maintained even in a community that is redundant with respect to each individual function. For example, though multiple populations may be capable of performing a function, they may not all perform it with the same efficiency, or they may not generate the same metabolic by-products. Similarly, a "replacement" species may not have the same growth rate or competitive ability as the original community member. Changes such as this could influence the activity of other populations in the community, and indirectly cause a change in overall ecosystem function, despite the fact that the original function of interest has been maintained (Chapin et al., 1997). More research is needed to determine whether the presence of multiple species, with overlapping functional abilities, actually results in functional stability. Further research into the relationship between microbial community structure and function, and the role of functional redundancy in these systems is an important first step toward determining whether it is possible to control microbial communities in a manner that will help scientists manage the stability, function, and quality of an ecosystem as a whole.

9.4. Final comments

While increased knowledge of the many factors influencing microbial community structure, and the role of space and time in this relationship, is important, there is particular interest in understanding how these factors may affect the activity of microorganisms in an ecosystem. Microorganisms play a fundamental role in establishing the biogeochemical cycles necessary for the long-term functioning of ecosystems. In addition, microorganisms are primarily responsible for the degradation and detoxification of many environmental contaminants. For these reasons, changes in the composition or activity of microbial communities can have immediate and lasting effects on ecosystem functioning.

Increased research into the relationship between structure and function of microbial communities is necessary before scientists can anticipate how habitat disruption and changes in community structure may impact community activity and ecosystem performance, and a better understanding of the role of spatial heterogeneity in microbial communities with help ecologists to determine the relevance of small-scale observations and experiments for large-scale patterns and processes. By characterizing the spatial structure of microbial communities, the scales at which factors controlling their development operate may be identified, thus shedding light on the nature of the factors themselves. Predictive relationships for assessing the impact of variables such as management practices or environmental changes on the microbial community, and thus microbial function, may then be improved.

Bibliography

Acosta, D. and D. H. Lynn. 2002. A preliminary assessment of spatial patterns of soil ciliate diversity in two subtropical forests in Puerto Rico and its implications for designing an appropriate sampling approach. Soil Biology and Biochemistry. 34:1517-1520.

Adrian, N. R., J. A. Robinson and J. M. Suflita. 1994. Spatial variability in biodegradation rates as evidenced by methane production from an aquifer. Applied and Environmental Microbiology. 60:3632-3639.

Aldén, L., F. Demoling and E. Bååth. 2001. Rapid method of determining factors limiting bacterial growth in soil. Applied and Environmental Microbiology. 67:1830-1838.

Allen, M. F. and J. A. MacMahon. 1985. Impact of disturbance on cold desert fungi: comparative microscale dispersion patterns. Pedobiologia. 28:215-224.

Amato, M. and J. N. Ladd. 1988. Assay for microbial biomass based on ninhydrin-reactive nitrogen in extracts of fumigated soils. Soil Biology and Biochemistry. 20:107-114.

Andrén, O., J. Bengtsson and M. Clarholm. 1995. Biodiversity and species redundancy among litter decomposers. *In:* Collins, H. P., G. P. Robertson and M. J. Klug (ed.), The significance and regulation of soil biodiversity. Netherlands, Kluwer Academic Publishers. pp 141-151.

Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiological Reviews. 45:180-209.

Atlas, R. M., A. Horowitz, M. Krichevsky and A. K. Bej. 1991. Response of microbial-populations to environmental disturbance. Microbial Ecology. 22:249-256.

Atlas, R. M. and R. Bartha. 1992. Hydrocarbon biodegredation and oil spill bioremediation. *In:* Marshall, K. C. (ed.), Advances in microbial ecology. New York, Plenum. 12:287-338.

Avois, C., P. Legendre, S. Masson and B. Pinel-Alloul. 2000. Is the sampling strategy interfering with the study of spatial variability of zooplankton communities? Canadian Journal of Fisheries and Aquatic Sciences. 57:1940-1956.

Azam, F. and J. W. Ammerman. 1984. Cycling of organic matter by bacterioplankton in pelagic marine ecosystems: microenvironmental considerations. *In:* Fasham, M. J. R. (ed.), Flows of energy and materials in marine ecosystems. New York, Plenum Press. pp 345-360.

Balkwill, D. L. 1989. Numbers, diversity, and morphological characteristics of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiology Journal. 7:33-52.

Balser, T. C. and M. K. Firestone. 1996. Sources of variability in BIOLOG assays of soil microbial communities: spatial and analytical. Abstracts of the Substrate Utilization for Characterization of Microbial Communities in Terrestrial Ecosystems Conference. Innsbruck, Austria.

Beare, M. H., D. C. Coleman, D. A. Crossley, P. F. Hendrix and E. P. Odum. 1995. A hierarchical approach to evaluating the significance of soil biodiversity to biogeochemical cycling. Plant and Soil. 170:5-22.

Beckett, P. H. T. and R. Webster. 1971. Soil variability: a review. Soils and Fertility. 34:1-15.

Bej, A. K. and M. H. Mahbubani. 1994. Applications of the polymerase chain reaction (PCR) in vitro DNA-amplification method in environmental microbiology. *In:* Griffin, H. G. and A. M. Griffin (ed.), PCR Technology: Current Innovations. Boca Raton, Florida. CRC Press. pp 327-339.

Bell, W. and R. Mitchell. 1972. Chemotactic and growth responses of marine bacteria to algal extracellular products. Biological Bulletin. 143265-277.

Beloin, R. M., J. L. Sinclair and W. C. Ghiorse. 1988. Distribution and activity of microorganisms in subsurface sediments in a pristine study site in Oklahoma. Microbial Ecology. 16:85-97.

Bending, G. D. and M. K. Turner. 1999. Interaction of biochemical quality and particle size of crop residues and its effect on the microbial biomass and nitrogen dynamics following incorporation into soil. Biology and Fertility of Soils. 29:319-327.

Bending, G. D., E. Shaw and A. Walker. 2001. Spatial heterogeneity in the metabolism and dynamics of isoproturon degrading microbial communities in soil. Biology and Fertility of Soils. 33:484-489.

Benedetti-Cecchi, L. 2001. Variability in abundance of algae and invertebrates at different spatial scales on rocky sea shores. Marine Ecology Progress Series. 215:79-92.

Bengtsson, G. 1989. Growth and metabolic flexibility in groundwater bacteria. Microbial Ecology. 18:235-248.

Berardesco, G., S. Dyhrman, E. Gallagher and M. P. Shiaris. 1998. Spatial and temporal variation of phenanthrene-degrading bacteria in intertidal sediments. Applied and Environmental Microbiology. 64:2560-2565.

Blackburn, N., T. Fenchel and J. Mitchell. 1998. Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science. 282:2254-2256.

Blackburn, N. and T. Fenchel. 1999. Influence of bacteria, diffusion and sheer on micro-scale nutrient patches, and implications for bacterial chemotaxis. Marine Ecology Progress Series. 189:1-7.

Blum, L. K., M. S. Roberts, J. L. Garland and A. L. Mills. 2004. Microbial communities among the dominant high marsh plants and associated sediments of the United States East Coast. Microbial Ecology. *In press.*

Blume, E., M. Bischoff, J. M. Reichert, T. Moorman, A. Konopka and R. F. Turco. 2002. Surface and subsurface microbial biomass, community structure and metabolic activity as a function of soil depth and season. Applied Soil Ecology. 20:171-181.

Bockelmann, U., W. Manz, T. R. Neu and U. Szewzyk. 2002. Investigation of lotic microbial aggregates by a combined technique of fluorescent *in situ* hybridization and lectin- binding-analysis. Journal of Microbiological Methods. 49:75-87.

Boerner, R. E. J. and S. D. Koslowsky. 1989. Microsite variations in soil chemistry and nitrogen mineralization in a beech-maple forest. Soil Biology and Biochemistry. 21:795-801.

Boerner, R. E. J. and J. Rebbeck. 1995. Decomposition and nitrogen release from leaves of three hardwood species grown under elevated O_3 and/or CO_2. *In:* Collins, H. P., G. P. Robertson and M. J. Klug (ed.), The significance and regulation of soil biodiversity. Netherlands, Kluwer Academic Publishers. pp 169-177.

Bone, T. L. and D. L. Balkwill. 1988. Morphological and cultural comparison of microorganisms in surface soil and subsurface sediments in a pristine study site in Oklahoma. Microbial Ecology. 16:49-64.

Boon, N., C. Marle, E. M. Top and W. Verstraete. 2000. Comparison of the spatial homogeneity of physico-chemical parameters and bacterial 16S rRNA genes in sediment samples from a dumping site for dredging sludge. Applied Microbiology and Biotechnology. 53:742-747.

Both, G. J., S. Gerards and H. J. Laanbroek. 1992. Temporal and spatial variation in the nitrite-oxidizing bacterial community of a grassland soil. FEMS Microbiology Ecology. 101:99-112.

Bottomley, P. J. 1994. Light microscopic methods for studying soil microorganisms. *In:* Weaver, R. W., S. Angle, P. J. Bottomley, D. F. Bezdicek, S. Smith, A. Tabitabai, and A. Wollum (ed.), Methods of soil analysis, Part 2 - Microbiological and biochemical properties. Madison, Wisconsin. Soil Science Society of America. pp 81-105.

Bringmark, E. 1989. Spatial variation in soil pH of beech forests in relation to buffering properties and soil depths. Oikos. 54:165-177.

Briones, A. and L. Raskin. 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current Opinion in Biotechnology. 14:270-276.

Britten, R. J., D. E. Grahan and B. R. Neufield. 1974. Analysis of repeating DNA sequences by reassociation. Methods in Enzymology. 29:363-406.

Brock, T. D. 1987. The study of microorganisms in situ: progress and problems. *In:* Fletcher, M., T. R. G. Gray and J. G. Jones (ed.), Ecology of microbial communities. Cambridge U.K., Cambridge University Press. pp 1-17.

Brodie, E., S. Edwards and N. Clipson. 2003. Soil fungal community structure in a temperate upland grassland soil. FEMS Microbiology Ecology. 45:105-114.

Bruce, K. D., W. D. Hiorns, J. L. Hobman, A. M. Osborn, P. Strike and D. A. Ritchie. 1992. Amplification of DNA from native populations of soil bacteria using polymerase chain reaction. Applied and Environmental Microbiology. 58:3413-3416.

Bruckner, A., E. Kandeler and C. Kampichler. 1999. Plot-scale spatial patterns of soil water content, pH, substrate-induced respiration and N mineralization in a temperate coniferous forest. Geoderma. 93:207-223.

Brunk, C. F., E. Avaniss-Aghajani and C. A. Brunk. 1996. A computer analysis of primer and probe hybridization potential with bacterial small-subunit rRNA sequences. Applied and Environmental Microbiology. 62:872-879.

Buckley, D. H. and T. M. Schmidt. 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecology. 42:11-21.

Busse, H. J., E. B. M. Denner and W. Lubitz. 1996. Classification and identification of bacteria: current approaches to an old problem. Overview of methods used in bacterial systematics. Journal of Biotechnology. 47:3-38.

Button, D. K., F. Schut, P. Quang, R. Martin and B. R. Robertson. 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Applied and Environmental Microbiology. 59:881-891.

Callaghan, A. V. 1999. Processes governing the distribution of NO_3^- in the Columbia Aquifer at a field site near Oyster, VA. Masters thesis, Department of Environmental Sciences, University of Virginia, Charlottesville, VA.

Castrignanò, A., L. Giugliarini, R. Risaliti and N. Martinelli. 2000. Study of spatial relationships among some soil physico-chemical properties of a field in central Italy using multivariate geostatistics. Geoderma. 97:39-60.

Cavigelli, M. A., G. P. Robertson and M. J. Klug. 1995. Fatty-acid methyl-ester (FAME) profiles as measures of soil microbial community structure. Plant and Soil. 170:99-113.

Chachaty, E., P. Saulnier, A. Martin, N. Mario and A. Andremont. 1994. Comparison of ribotyping, pulse-field gel electrophoresis and random amplified polymorphic DNA for typing *Clostridium difficile* strains. FEMS Microbiology Letters. 122:61-68.

Chandler, D. P., S.-M. Li, C. M. Spadoni, G. R. Drake, D. L. Balkwill, J. K. Fredrickson and F. J. Brockman. 1997. A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from deep subsurface sediment. FEMS Microbiology Ecology. 23:131-144.

Chapelle, F. H. and D. R. Lovley. 1992. Competitive exclusion of sulfate-reduction by Fe III-reducing bacteria: A mechanism for producing discrete zones of high-iron ground water. Ground Water. 30:29-36.

Chapelle, F. H. 1993. Ground-water microbiology and geochemistry. New York, John Wiley & Sons, Inc.

Chapin, F. S., III, B. H. Walker, R. J. Hobbs, D. U. Hooper, J. H. Lawton, O. E. Sala and D. Tilman. 1997. Biotic control over the functioning of ecosystems. Science. 277:500-503.

Cho, J. C. and J. M. Tiedje. 2000. Biogeography and degree of endemicity of fluorescent Pseudomonas strains in soil. Applied and Environmental Microbiology. 66:5448-5456.

Cliff, A. D. and J. K. Ord. 1981. Spatial processes: models and applications. London, Pion Limited.

Colwell, F. S. and R. M. Lehman. 1997. Carbon source utilization profiles for microbial communities from hydrologically distinct zones of a basalt aquifer. Microbial Ecology. 33:240-251.

Connon, S. A. and S. J. Giovannoni. 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Applied and Environmental Microbiology. 68:3878-3885.

Cotrufo, M. F., P. Ineson and A. P. Rowland. 1994. Decomposition of tree leaf litters grown under elevated CO_2: effect of litter quality. Plant and Soil. 163:121-130.

Curtis, T. P., W. T. Sloan and J. W. Scannell. 2002. Estimating prokaryotic diversity and its limits. Proceedings of the National Academy of Sciences. 99:10494-10499.

Dahllof, I. 2002. Molecular community analysis of microbial diversity. Current Opinion in Biotechnology. 13:213-217.

Dandurand, L. M., G. R. Knudsen and D. J. Schotzko. 1995. Quantification of *Pythium-Ultimum Var Sporangiiferum* zoospore encystment patterns using geostatistics. Phytopathology. 85:186-190.

Dandurand, L. M., D. J. Schotzko and G. R. Knudsen. 1997. Spatial patterns of rhizoplane populations of *Pseudomonas fluorescens*. Applied and Environmental Microbiology. 63:3211-3217.

Danovaro, R., M. Armeni, A. Dell'Anno, M. Fabiano, E. Manini, D. Marrale, A. Pusceddu and S. Vanucci. 2001. Small-scale distribution of bacteria, enzymatic activities, and organic matter in coastal sediments. Microbial Ecology. 42:177-185.

Davin-Regli, A., Y. Abed, R. N. Charrel, C. Bollet and P. de Micco. 1995. Variations in DNA concentrations significantly affect the reproducibility of RAPD fingerprint patterns. Research in Microbiology. 146:561-568.

Davis, L. G., W. M. Kuehl and J. F. Battey. 1994. Amplification of DNA using the polymerase chain reaction (PCR). *In:* Davis, L. G., W. H. Kuehl and J. F. Battey (ed.), Basic Methods in Molecular Biology. East Norwalk, Connecticut. Appleton and Lange.

Dechesne, A., C. Pallud, D. Debouzie, J. P. Flandrois, T. M. Vogel, J. P. Gaudet and G. L. Grundmann. 2003. A novel method for characterizing the microscale 3D spatial distribution of bacteria in soil. Soil Biology and Biochemistry. 35:1537-1546.

Degens, B. P. 1998. Decreases in microbial functional diversity do not result in corresponding changes in decomposition under different moisture conditions. Soil Biology and Biochemistry. 30:1989-2000.

Degens, B. P. 1999. Catabolic response profiles differ between microorganisms grown in soils. Soil Biology and Biochemistry. 31:475-477.

Degens, B. P., L. A. Schipper, G. P. Sparling and L. C. Duncan. 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biology and Biochemistry. 33:1143-1153.

Demeke, T. and R. P. Adams. 1994. The use of PCR-RAPD analysis of plant taxonomy and evolution. *In:* Griffin, H. G. and A. M. Griffin (ed.), PCR Technology: Current Innovations. Boca Raton, Florida. CRC Press. pp 179-191.

Devereux, R. and S. G. Willis. 1995. Amplification of ribosomal RNA sequences. *In:* Akkermans, A. D. L., J. D. Van Elsas, and F. J. De Bruijn (ed.), Molecular Microbial Ecology Manual. Dordrecht, Netherlands. Kluwer Academic Publishers. pp 3.3.1:1-11.

Dickens, H. E. and J. M. Anderson. 1999. Manipulation of soil microbial community structure in bog and forest soils using chloroform fumigation. Soil Biology and Biochemistry. 31:2049-2058.

Dobermann, A., P. Goovaerts and T. George. 1995. Sources of soil variation in an acid Ultisol of the Philippines. Geoderma. 68:173-191.

Duarte, C. M. and D. Vaqué. 1992. Scale dependence of bacterioplankton patchiness. Marine Ecology Progress Series. 84:95-100.

Dunbar, J., L. O. Ticknor and C. R. Kuske. 2000. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Applied and Environmental Microbiology. 66:2943-2950.

Dutilleul, P. 1993. Spatial heterogeneity and the design of ecological field experiments. Ecology. 74:1646-1658.

Dutilleul, P. and B. Pinel-Alloul. 1996. A doubly multivariate model for statistical analysis of spatio- temporal environmental data. Environmetrics. 7:551-565.

Dutilleul, P., J. D. Stockwell, D. Frigon and P. Legendre. 2000. The Mantel test versus Pearson's correlation analysis: Assessment of the differences for biological and environmental studies. Journal of Agricultural Biological and Environmental Statistics. 5:131-150.

Eddison, J. C. and J. G. Ollason. 1978. Diversity in constant and fluctuating environments. Nature. 275:309-310.

Ellingsoe, P. and K. Johnsen. 2002. Influence of soil sample sizes on the assessment of bacterial community structure. Soil Biology and Biochemistry. 34:1701-1707.

Ellsworth, D. L., K. D. Rittenhouse and R. L. Honeycutt. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. BioTechniques. 14:214-216.

Elton, C. S. 1958. The ecology of invasions by plants and animals. London, Chapman and Hall.

Emmerling, C., T. Udelhoven and D. Schroder. 2001. Response of soil microbial biomass and activity to agricultural de-intensification over a 10-year period. Soil Biology and Biochemistry. 33:2105-2114.

Englund, E. and A. Sparks. 1991. GEOEAS 1.2.1.: Geostatistical Environmental Assessment Software User's Guide. Environmental Monitoring Systems Laboratory, United States Environmental Protection Agency. Las Vegas, Nevada.

Ettema, C. H. and D. A. Wardle. 2002. Spatial soil ecology. Trends in Ecology & Evolution. 17:177-183.

Ettema, C. H. and G. W. Yeates. 2003. Nested spatial biodiversity patterns of nematode genera in a New Zealand forest and pasture soil. Soil Biology and Biochemistry. 35:339-342.

Federle, T. W., R. M. Ventullo and D. C. White. 1990. Spatial distribution of microbial biomass, activity, community structure, and biodegradation of linear alkylbezene sulfonate (LAS) and linear alcohol ethoxylate (LAE) in the subsurface. Microbial Ecology. 20:297-313.

Felsenstein, J. 1993. PHYLIP. Phylogeny Inference Package. Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Felske, A. and A. D. L. Akkermans. 1998. Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soils. Microbial Ecology. 36:31-36.

Fenchel, T. 1984. Suspended marine bacteria as a food source. *In:* Fasham, M. J. R. (ed.), Flows of energy and materials in marine ecosystems. New York, Plenum Press. pp 301-316.

Fendorf, S. E., G. C. Li, M. J. Morra and L. M. Dandurand. 1997. Imaging a pseudomonad in mineral suspensions with scanning force and electron microscopy. Soil Science Society of America Journal. 61:109-115.

Fernandez, A., S. Y. Huang, S. Seston, J. Xing, R. Hickey, C. Criddle and J. Tiedje. 1999. How stable is stable? Function versus community composition. Applied and Environmental Microbiology. 65:3697-3704.

Ferris, M. J., G. Muyzer and D. M. Ward. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA- defined populations inhabiting a hot spring microbial mat community. Applied and Environmental Microbiology. 62:340-346.

Fierer, N., J. P. Schimel and P. A. Holden. 2003. Variations in microbial community composition through two soil depth profiles. Soil Biology and Biochemistry. 35:167-176.

Finlay, B. J., J. O. Corliss, G. Esteban and T. Fenchel. 1996. Biodiversity at the microbial level: The number of free-living ciliates in the biosphere. Quarterly Review of Biology. 71:221-237.

Finlay, B. J., S. C. Maberly and J. I. Cooper. 1997. Microbial diversity and ecosystem function. Oikos. 80:209-213.

Finlay, B. J., G. F. Esteban, J. L. Olmo and P. A. Tyler. 1999. Global distribution of free-living microbial species. Ecography. 22:138-144.

Finlay, B. J., G. F. Esteban, K. J. Clarke and J. L. Olmo. 2001. Biodiversity of terrestrial protozoa appears homogeneous across local and global spatial scales. Protist. 152:355-366.

Franklin, R. B., D. R. Taylor and A. L. Mills. 1999 a. Characterization of microbial communities using randomly amplified polymorphic DNA (RAPD) Journal of Microbiological Methods. 35:225-235.

Franklin, R. B., D. R. Taylor and A. L. Mills. 1999 b. The distribution of microbial communities in anaerobic and aerobic zones of a shallow coastal plain aquifer. Microbial Ecology. 38:377-386.

Franklin, R. B. and A. L. Mills. 2001. Spatial distribution of microbial community structure in an agricultural wheat field: fine scale variability and patterns. Ninth International Symposium on Microbial Ecology, Amsterdam, the Netherlands

Franklin, R. B., J. L. Garland, C. H. Bolster and A. L. Mills. 2001. Impact of dilution on microbial community structure and functional potential: Comparison of numerical simulations and batch culture experiments. Applied and Environmental Microbiology. 67:702-712.

Franklin, R. B., L. K. Blum, A. C. McComb and A. L. Mills. 2002. A geostatistical analysis of small-scale spatial variability in bacterial abundance and community structure in salt marsh creek bank sediments. FEMS Microbiology Ecology. 42:71-80.

Franklin, R. B. and A. L. Mills. 2003. Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. FEMS Microbiology Ecology. 44:335-346.

Frischer, M. E., J. M. Danforth, M. A. N. Healy and F. M. Saunders. 2000. Whole-cell versus total RNA extraction for analysis of microbial community structure with 16S rRNA-targeted oligonucleotide probes in salt marsh sediments. Applied and Environmental Microbiology. 66:3037-3043.

Fuhrman, J. A., D. E. Comeau, A. Hagstrom and A. M. Chan. 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Applied and Environmental Microbiology. 57:2351-2359.

Fulthorpe, R. R., A. N. Rhodes and J. M. Tiedje. 1998. High levels of endemicity of 3-chlorobenzoate-degrading soil bacteria. Applied and Environmental Microbiology. 64:1620-1627.

Garcia-Pichel, F., L. Prufert-Bebout and G. Muyzer. 1996. Phenotypic and phylogenetic analyses show *Microcoleus chthonoplastes* to be a cosmopolitan cyanobacterium. Applied and Environmental Microbiology. 62:3284-3291.

Garland, J. L. and A. L. Mills. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Applied and Environmental Microbiology. 57:2351-2359.

Garland, J. L. 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biology and Biochemistry. 28:213-221.

Garland, J. L. and R. M. Lehman. 1999. Dilution/extinction of community phenotypic characters to estimate relative structural diversity in mixed communities. FEMS Microbiology Ecology. 30:333-343.

Garland, J. L., A. L. Mills, A. Morales and K. Cook. 1999. Survival of human-associated bacteria in prototype advanced life support systems. SAE Technical Paper Series No. 1999-01-2601.

Ghilarov, A. M. 1997. Species redundancy versus non-redundancy: Is it worth further discussion? Zhurnal Obshchei Biologii. 58:100-105.

Ghiorse, W. C. and J. T. Wilson. 1988. Microbial ecology of the terrestrial subsurface. Advanced Applied Microbiology. 33:107-172.

Gilbert, J., D. L. Danielopol and J. A. Stanford. 1994. Groundwater Ecology. San Diego, California, Academic Press.

Giller, K. E., M. H. Beare, P. Lavelle, A.-M. N. Izac and M. J. Swift. 1997. Agricultural intensification, soil biodiversity and agroecosystem function. Applied Soil Ecology. 6:3-16.

Giller, P. S. 1984. Community structure and the niche. London, Chapman and Hall.

Gitay, H., J. B. Wilson and W. G. Lee. 1996. Species redundancy: A redundant concept? Journal of Ecology. 84:121-124.

Gonod, L. V., C. Chenu and G. Soulas. 2003. Spatial variability of 2,4-dichlorophenoxyacetic acid (2,4-D) mineralization potential at a millimetre scale in soil. Soil Biology and Biochemistry. 35:373-382.

Goovaerts, P. 1998. Geostatistical tools for characterizing the spatial variability of microbiological and physico-chemical soil properties. Biology and Fertility of Soils. 27:315-334.

Gorres, J. H., M. J. Dichiaro, J. B. Lyons and J. A. Amador. 1998. Spatial and temporal patterns of soil biological activity in a forest and an old field. Soil Biology and Biochemistry. 30:219-230.

Gounot, A. M. 1994. Microbial ecology of groundwaters. *In:* Gilbert, J., D. L. Danielopol and J. A. Stanford (ed.), Groundwater Ecology. San Diego, California. Academic Press. pp 189-215.

Gower, J. C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika. 53:325-338.

Gower, J. C. 1971. A general coefficient of similarity and some of its properties. Biometrics. 27:857-871.

Grayston, S. J., G. S. Griffith, J. L. Mawdsley, C. D. Campbell and R. D. Bardgett. 2001. Accounting for variability in soil microbial communities of temperate upland grassland ecosystems. Soil Biology and Biochemistry. 33:533-551.

Griffiths, B. S., K. Ritz and L. A. Glover. 1996. Broad-scale approaches to the determination of soil microbial community structure: Application of the community DNA hybridization technique. Microbial Ecology. 31:269-280.

Griffiths, B. S., K. Ritz and R. E. Wheatley. 1997. Relationship between functional diversity and genetic diversity in complex microbial communities. *In:* Insam, H. and A. Rangger (ed.), Microbial Communities: Functional versus structural approaches. Germany, Springer-Verlag. pp 1-9.

Griffiths, B. S., K. Ritz, R. D. Bardgett, R. Cook, S. Christensen, F. Ekelund, S. J. Sorensen, E. Baath, J. Bloem, P. C. de Ruiter, J. Dolfing and B. Nicolardot. 2000. Ecosystem response of pasture soil communities to fumigation- induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. Oikos. 90:279-294.

Griffiths, B. S., K. Ritz, R. Wheatley, H. L. Kuan, B. Boag, S. Christensen, F. Ekelund, S. J. Sorensen, S. Muller and J. Bloem. 2001. An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. Soil Biology and Biochemistry. 33:1713-1722.

Griffiths, R. I., A. S. Whiteley, A. G. O'Donnell and M. J. Bailey. 2003. Influence of depth and sampling time on bacterial community structure in an upland grassland soil. FEMS Microbiology Ecology. 43:35-43.

Grime, J. P. 1997. Biodiversity and ecosystem function: the debate deepens. Science. 277:1260-1261.

Grundmann, L. G. and F. Gourbiere. 1999. A micro-sampling approach to improve the inventory of bacterial diversity in soil. Applied Soil Ecology. 13:123-126.

Grundmann, G. L. and D. Debouzie. 2000. Geostatistical analysis of the distribution of NH_4^+ and NO_2^- - oxidizing bacteria and serotypes at the millimeter scale along a soil transect. FEMS Microbiology Ecology. 34:57-62.

Grundmann, G. L., A. Dechesne, F. Bartoli, J. P. Flandrois, J. L. Chasse and R. Kizungu. 2001. Spatial modeling of nitrifier microhabitats in soil. Soil Science Society of America Journal. 65:1709-1716.

Guggenheim, M., S. Shapiro, R. Gmur and B. Guggenheim. 2001. Spatial arrangements and associative behavior of species in an in vitro oral biofilm model. Applied and Environmental Microbiology. 67:1343-1350.

Hackney, C. T. and A. A. De La Cruz. 1980. *In situ* decomposition of roots and rhizomes of two tidal marsh plants. Ecology. 61:226-231.

Hackney, C. T. and A. A. De La Cruz. 1986. Belowground productivity of roots and rhizomes in a giant cordgrass (*Spartina cynosuroides)* marsh. Estuaries. 9:112-116.

Haldeman, D. L. and P. S. Amy. 1993. Diversity within a colony morphotype: Implications for ecological research. Applied and Environmental Microbiology. 59:933-935.

Handelsman, J. and K. Smalla. 2003. Techniques: Conversations with the silent majority. Current Opinion in Microbiology. 6:271-273.

Harris, P. J. 1994. Consequences of the spatial distribution of microbial communities in soil. *In:* Ritz, R., J. Kighton and K. E. Giller (ed.), Beyond the Biomass. New York, British Society of Soil Science, Wiley-Sayce Publishers.

Head, I. M., F. M. Saunders and R. W. Pickup. 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial Ecology. 35:1-21.

Hewitt, J. E., S. F. Thrush, V. J. Cummings and S. J. Turner. 1998. The effect of changing sampling scales on our ability to detect effects of large-scale processes on communities. Journal of Experimental Marine Biology and Ecology. 227:251-264.

Hill, I., James and R. G. Wiegert. 1980. Microcosms in ecological modeling. Microcosms in ecological research. U.S. Department of Energy, Symp. 52, Springfield, VA, National Information Service.

Hines, M. E., R. S. Evans, B. R. Sharak Genthner, S. G. Willis, S. Friedman, J. N. Rooney-Varga and R. Devereux. 1999. Molecular phylogenetic and biogeochemical studies of sulfate-reducing bacteria in the rhizosphere of *Spartina alterniflora*. Applied and Environmental Microbiology. 65:2209-2216.

Hobbie, J. E., R. J. Daley and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescent microscopy. Applied and Environmental Microbiology. 33:1225-1228.

Holben, W. E. and D. Harris. 1995. DNA-based monitoring of total bacterial community structure in environmental samples. Molecular Ecology. 4:627-631.

Holben, W. E. 1997. Isolation and purification of bacterial community DNA from environmental samples. *In:* Hurst, C. J., G. R. Knudsen, M. J. McInerney, L. D. Stetzenback and M. V. Walter (ed.), Manual of environmental microbiology. Washington, D. C., American Society for Microbiology Press. pp 431-444.

Hooper, D. U. and P. M. Vitousek. 1997. The effects of plant composition and diversity on ecosystem processes. Science 277:1302-1305.

Hughes, J. B. and O. L. Petchey. 2001. Merging perspectives on biodiversity and ecosystem functioning. Trends in Ecology & Evolution. 16:222-223.

Hughes, J. B., J. J. Hellmann, T. H. Ricketts and B. J. M. Bohannan. 2001. Counting the uncountable: Statistical approaches to estimating microbial diversity. Applied and Environmental Microbiology 67:4399-4406.

Hutchinson, G. E. 1959. Homage to Santa Rosalia, or why are there so many kinds of animals? American Naturalist 93:145-159.

Isaaks, E. H. and R. M. Srivastava. 1989. An introduction to applied geostatistics. New York, Oxford University Press.

Jaspers, E., K. Nauhaus, H. Cypionka and J. Overmann. 2001. Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. FEMS Microbiology Ecology. 36:153-64.

Jensen, E. S. 1994. Mineralization-immobilization of nitrogen in soil amended with low C:N plant residues with different particle sizes. Soil Biology and Biochemistry. 26:519-521.

Jiménez, L., G. L. deVictoria, J. Wear, C. B. Fliermans and T. C. Hazen. 1990. Molecular analysis of deep subsurface bacteria. *In:* Fliermans, C. B. and T. C. Hazen (ed.), Microbiology of the deep subsurface. Aiken, South Carolina. WSRC Information Services S2 97-113.

Johnsen, K., C. S. Jacobsen, V. Torsvik and J. Sorensen. 2001. Pesticide effects on bacterial diversity in agricultural soils - A review. Biology and Fertility of Soils. 33:443-453.

Jordan, F. L. and R. M. Maier. 1999. Development of an agar lift-DNA/DNA hybridization technique for use in visualization of the spatial distribution of Eubacteria on soil surfaces. Journal of Microbiological Methods. 38:107-117.

Juergens, K., H. Arndt and H. Zimmermann. 1997. Impact of metazoan and protozoan grazers on bacterial biomass distribution in microcosm experiments. Aquatic Microbial Ecology. 12:131-138.

Kandeler, E., C. Kampichler and O. Horak. 1996. Influence of heavy metals on the functional diversity of soil microbial communities. Biology and Fertility of Soils. 23:299-306.

Karp, A., O. Seberg and M. Buiatti. 1996. Molecular techniques in the assessment of botanical diversity. Annals of Botany. 78:143-149.

Kawaguchi, T. and A. W. Decho. 2002. *In situ* microspatial imaging using two-photon and confocal laser scanning microscopy of bacteria and extracellular polymeric secretions (EPS) within marine stromatolites. Marine Biotechnology. 4:127-131.

Kennedy, A. C. 1995. Carbon utilization and fatty acid profiles for characterization of bacteria. *In:* Weaver, R. and J. S. Angle. Methods of Soil Analysis, Part 2: Microbiological and biochemical properties. Madison, Wisconsin. Soil Science Society of America.

Kennedy, A. C. and K. L. Smith. 1995. Soil microbial diversity and the sustainability of agricultural soils. Plant and Soil. 170:75-86.

Kennedy, A. C. and V. L. Gewin. 1997. Soil microbial diversity: Present and future considerations. Soil Science 162:607-617.

Kettler, T. A., J. W. Doran and T. L. Gilbert. 2001. Simplified method for soil particle-size determination to accompany soil-quality analyses. Soil Science Society of America Journal. 65:849-852.

Kieft, T. L., E. Wilch, K. O'Connor, D. B. Ringelberg and D. C. White. 1997. Survival and phospholipid fatty acid profiles of surface and subsurface bacteria in natural sediment microcosms. Applied and Environmental Microbiology. 63:1531-1542.

King, G. M.. 2002. Radiotracer ^{14}C and ^{3}H assays for benthic microbial organic matter transformations. *In:* Hurst, C. J., R. L. Crawford, G. R. Knudsen, M. J. McInerney, and L. D. Stetzenback (ed.), Manual of environmental microbiology. Washington, D. C., American Society for Microbiology Press. pp 419-426.

Kirchman, D. L., L. Y. Yu, B. M. Fuchs and R. Amann. 2001. Structure of bacterial communities in aquatic systems as revealed by filter PCR. Aquatic Microbial Ecology. 26:13-22.

Klein, D. A., W. C. Metzger, B. A. Frederick and E. F. Redente. 1986. Environmental stress-functional diversity relationships in semiarid terrestrial microbial communities. *In:* Jensen, V., A. Kjoller and L. H. Sorensen (ed.), Microbial communities in soil. London. Elsevier Applied Science Publishers. pp 105-112.

Klironomos, J. N., M. C. Rillig and M. F. Allen. 1999. Designing belowground field experiments with the help of semi-variance and power analyses. Applied Soil Ecology. 12:227-238.

Knapp, E. P. 1997. The influence of redox conditions on sorption to aquifer sediments: transport of reactive solutes in groundwater. Ph.D. Dissertation, Department of Environmental Sciences, University of Virginia, Charlottesville, VA.

Konopka, A., L. Oliver and R. F. Turco. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. Microbial Ecology. 35:103-115.

Kourtev, P. S., J. G. Ehrenfeld and M. Haggblom. 2003. Experimental analysis of the effect of exotic and native plant species on the structure and function of soil microbial communities. Soil Biology and Biochemistry. 35:895-905.

Kozdroj, J. and J. D. van Elsas. 2001. Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. Journal of Microbiological Methods. 43:197-212.

Krembs, C., A. R. Juhl, R. A. Long and F. Azam. 1998a. Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. Limnology and Oceanography. 43:307-314.

Krembs, C., A. R. Juhl and J. R. Strickler. 1998b. The spatial information preservation method: Sampling the nanoscale spatial distribution of microorganisms. Limnology and Oceanography 43:298-306.

Kristensen, H. L., K. Debosz and G. W. McCarty. 2003. Short-term effects of tillage on mineralization of nitrogen and carbon in soil. Soil Biology and Biochemistry. 35:979-986.

Kuperman, R. G., G. P. Williams and R. W. Parmelee. 1998. Spatial variability in the soil foodwebs in a contaminated grassland ecosystem. Applied Soil Ecology. 9:509-514.

Kuzyakova, I. F., V. A. Romanenkov and Y. V. Kuzyakov. 2001. Application of geostatistics in processing the results of soil and agrochemical studies. Eurasian Soil Science. 34:1219-1228.

Laczko, E., A. Rudaz and M. Aragno. 1997. Diversity of anthropogenically influenced or disturbed microbial communities. *In:* Insam, H. and A. Rangger (ed.), Microbial Communities: Functional versus structural approaches. Germany, Springer-Verlag.

Lambers, H. 1993. Rising CO_2, secondary plant metabolism, plant-herbivore interactions and litter decomposition: theoretical considerations. Vegetatio. 104/105:263-271.

Lancaster, L. and A. L. Mills. 1995. Structure and activity of subsurface microbial communities in a sandy coastal plain aquifer. Abstracts of the 95th General Meeting of the American Society for Microbiology.

Lavigne, C., S. K. Juniper and N. Silverberg. 1997. Spatio-temporal variability in benthic microbial activity and particle flux in the Laurentian Trough. Deep-Sea Research Part I-Oceanographic Research Papers. 44:1793-1813.

Lawton, J. H. 1994. What do species do in ecosystems? Oikos. 71:367-374.

Lebaron, P., J. F. Ghiglione, C. Fajon, N. Batailler and P. Normand. 1998. Phenotypic and genetic diversity within a colony morphotype. FEMS Microbiology Letters. 160:137-143.

Lee, S. and J. A. Fuhrman. 1990. DNA hybridization to compare species composition of natural bacterioplankton assemblages. Applied and Environmental Microbiology. 56:739-746.

Lee, S. and J. A. Fuhrman. 1991. Spatial and temporal variation of natural bacterioplankton assemblages studied by total genomic DNA cross-hybridization. Limnology and Oceanography. 36:1277-1287.

Legendre, P. and M. Troussellier. 1988. Aquatic heterotrophic bacteria - modeling in the presence of spatial auto-correlation. Limnology and Oceanography. 33:1055-1067.

Legendre, P. and M. J. Fortin. 1989. Spatial pattern and ecological analysis. Vegetatio. 80:107-138.

Legendre, P., M. Troussellier, V. Jarry and M. J. Fortin. 1989. Design for simultaneous sampling of ecological variables - from concepts to numerical-solutions. Oikos. 55:30-42.

Legendre, P., N. L. Oden, R. R. Sokal, A. Vaudor and J. Kim. 1990. Approximate analysis of variance of spatially autocorrelated regional data. Journal of Classification. 7:53-75.

Legendre, P. and A. Vaudor. 1991. The R package: Multidimensional analysis, spatial analysis. Montreal, Department of biological sciences, University of Montreal.

Legendre, P. 1993. Spatial autocorrelation - trouble or new paradigm. Ecology. 74:1659-1673.

Legendre, P. and L. Legendre. 1998. Numerical ecology. Amsterdam, Elsevier Scientific Publishing Company.

Lehman, R. M., F. S. Colwell, D. B. Ringelberg and D. C. White. 1995. Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. Journal of Microbiological Methods. 22:263-281.

Lehman, J. T. and D. Scavia. 1982. Microscale patchiness of nutrients in plankton communities. Science. 216:729-730.

Levin, S. A. 1992. The problem of pattern and scale in ecology. Ecology. 73:1943-1967.

Levin, S. A. and L. A. Segal. 1976. Hypothesis for origin of planktonic patchiness. Nature. 259:659.

Licht, T. R., K. A. Krogfelt, P. S. Cohen, L. K. Poulsen, J. Urbance and S. Molin. 1996. Role of lipopolysaccharide in colonization of the mouse intestine by *Salmonella typhimurium* studied by *in situ* hybridization. Infection and Immunity. 64:3811-3817.

Lin, A. W., M. A. Usera, T. J. Barrett and R. A. Goldsby. 1996. Application of random amplified polymorphic DNA analysis to differential strains of *Salmonella enteritidis.* Journal of Clinical Microbiology. 34:870-876.

Linton, D., F. E. Dewhirst, J. P. Clewley, R. J. Owen, A. P. Burnens and J. Stanley. 1994. Two types of 16S ribosomal-RNA gene are found in Campylobacter- Helveticus - analysis, applications and characterization of the intervening sequence found in some strains. Microbiology-UK. 140:847-855.

Liu, W., T. L. Marsh, H. Cheng and L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology. 63:4516-4522.

Liu, W. T., T. L. Marsh and L. J. Forney. 1998. Determination of the microbial diversity of anaerobic-aerobic activated sludge by a novel molecular biological technique. Water Science and Technology. 37:417-422.

Long, R. A. and F. Azam. 2001. Microscale patchiness of bacterioplankton assemblage richness in seawater. Aquatic Microbial Ecology. 26:103-113.

Long, S. C., C. M. Aelion, D. C. Dobbins and F. K. Pfaender. 1995. A comparison of microbial community characteristics among petroleum-contaminated and uncontaminated subsurface soil samples. Microbial Ecology. 30:297-307.

Lovell, R. D. and S. C. Jarvis. 1998. Soil microbial biomass and activity in soil from different grassland management treatments stored under controlled conditions. Soil Biology and Biochemistry. 30:2077-2085.

Lowit, M. B., L. K. Blum and A. L. Mills. 1998. Spatial and temporal variability of microbial communities in estuarine ecosystems. Abstracts of the 98th General Meeting of the American Society for Microbiology, Atlanta, Georgia.

MacArthur, R. H. 1957. On the relative abundance of bird species. Proceedings of the National Academy of Sciences. 43:293-295.

Mackas, D. L. 1984. Spatial autocorrelation of plankton community composition in a continental shelf ecosystem. Limnology and Oceanography. 29:451-471.

MacMillin, K. M., L. K. Blum and A. L. Mills. 1992. Comparison of bacterial dynamics in tidal creeks of the lower Delmarva Peninsula, Virginia, USA. Marine Ecology Progress Series. 86:110-121.

Macnaughton, S. J., T. Booth, T. M. Embley and A. G. O'Donnell. 1996. Physical stabilization and confocual microscopy of bacteria on roots using 16S rRNA targeted, fluorescent-labelled oligonucleotide probes. Journal of Microbiological Methods. 26279-285.

Madsen, E. L. and W. C. Ghiorse. 1993. Groundwater microbiology: subsurface ecosystem processes. *In:* Ford, T. E. (ed.), Aquatic microbiology: an ecological approach. Boston, MA. Blackwell Publishing Company. pp 167-213.

Magurran, A. E. 1988. Ecological diversity and its measurement. Princeton, NJ. Princeton University Press.

Makino, S.-I., Y. Okada, T. Karuyama, S. Kaneko and C. Sasakawa. 1994. PCR-based random amplified polymophic DNA fingerprinting of *Yersinia pseudotuberculosis* and its practical applications. Journal of Clinical Microbiology. 32:65-69.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research. 27:209-220.

Manz, W., G. Arp, G. Schumann-Kindel, U. Szewzyk and J. Reitner. 2000. Widefield deconvolution epifluorescence microscopy combined with fluorescence *in situ* hybridization reveals the spatial arrangement of bacteria in sponge tissue. Journal of Microbiological Methods. 40:125-134.

Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Current Opinions in Microbiology. 2:323-327.

Marxsen, J. 1988. Investigations into the number of respiring bacteria in groundwater from sandy and gravelly deposits. Microbial Ecology. 16:65-72.

Massol-Deya, A. A., D. A. Odelson, R. F. Hickey and J. M. Tiedje. 1995. Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis. ARDRA. *In:* Akkermans, A. D. L., J. D. Van Elsas, and F. J. De Bruijn (ed.), Molecular Microbial Ecology Manual. Dordrecht, Netherlands. Kluwer Academic Publishers. pp 3.3.2:1-8.

McGrady-Steed, J., P. M. Harris and P. J. Morin. 1997. Biodiversity regulates ecosystem predictability. Nature 390:162-165.

McNaughton, S. J. 1977. Diversity and stability of ecological communities: a comment on the role of empiricism in ecology. The American Naturalist. 111:515-525.

Meunier, J. R. and P. A. D. Grimont. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Research in Microbiology. 144:373-379.

Mikola, J. and H. Setala. 1998. Relating species diversity to ecosystem functioning: mechanistic backgrounds and experimental approach with a decomposer food web. Oikos. 83:180-194.

Miller, M. 1999. MANTEL-STRUCT: A program for the detection of population structure via Mantel tests. Journal of Heredity. 90:258-259.

Mills, A. L. and L. M. Mallory. 1987. The community structure of sessile heterotrophic bacteria stressed by acid mine drainage. Microbial Ecology. 14:219-232.

Mills, A. L., J. S. Herman, G. M. Hornberger and R. M. Ford. 2003. Functional redundancy promotes functional stability in diverse microbial bioreactor communities. SAE Paper Number 2003-01-2509.

Mitchell, J. G. and J. A. Fuhrman. 1989. Centimeter scale vertical heterogeneity is bacteria and chlorophyll *a*. Marine Ecology Progress Series. 54:141-148.

Moll, D. M., R. S. Summers and A. Breen. 1998. Microbial characterization of biological filters used for drinking water treatment. Applied and Environmental Microbiology. 64:2755-2759.

Morales, A., J. L. Garland and D. V. Lim. 1996. Survival of potentially pathogenic human-associated bacteria in the rhizosphere of hydroponically grown wheat. FEMS Microbiology Ecology. 20:155-162.

Moran, M. A., A. E. Maccubbin, R. Benner and R. E. Hodson. 1987. Dynamics of microbial biomass and activity in five habitats of the Okefenokee Swamp ecosystem. Microbial Ecology. 14:203-217.

Morris, S. J. and R. E. J. Boerner. 1998. Landscape patterns of nitrogen mineralization and nitrification in southern Ohio hardwood forests. Landscape ecology. 13:215-224.

Morris, S. J. 1999. Spatial distribution of fungal and bacterial biomass in southern Ohio hardwood forest soils: fine scale variability and microscale patterns. Soil Biology and Biochemistry. 31:1375-1386.

Morris, S. J. and R. E. J. Boerner. 1999. Spatial distribution of fungal and bacterial biomass in southern Ohio hardwood forest soils: scale dependency and landscape patterns. Soil Biology and Biochemistry. 31:887-902.

Mottonen, M., E. Jarvinen, T. J. Hokkanen, T. Kuuluvainen and R. Ohtonen. 1999. Spatial distribution of soil ergosterol in the organic layer of a mature Scots pine *(Pinus sylvestris L.)* forest. Soil Biology and Biochemistry. 31:503-516.

Muller-Niklas, G., M. Agis and G. J. Herndl. 1996. Microscale distribution of bacterioplankton in relation to phytoplankton: results from 100-nl samples. Limnology and Oceanography. 41:1577-1582.

Murray, C. J. 2001. Sampling and data analysis for environmental microbiology. *In:* Hurst, C. J., R. L. Crawford, G. R. Knudsen, M. J. McInerney, and L. D. Stetzenback (ed.), Manual of environmental microbiology. Washington, D. C., American Society for Microbiology Press. pp 166-177.

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

Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural systems. Current Opinions in Microbiology. 2:317-322.

Naeem, S., L. J. Thompson, S. P. Lawler, J. H. Lawton and R. M. Woodfin. 1994. Declining biodiversity can alter the performance of ecosystems. Nature. 368:734-737.

Naeem, S. and S. B. Li. 1997. Biodiversity enhances ecosystem reliability. Nature. 390:507-509.

Ninet, B., M. Monod, S. Emler, J. Pawlowski, C. Metral, P. Rohner, R. Auckenthaler and B. Hirschel. 1996. Two different 16S rRNA genes in a mycobacterial strain. Journal of Clinical Microbiology. 34:2531-2536.

Norusis, M. J. 1994. SPSS Professional Statistics 6.1. Chicago, IL, SPSS Inc.

Nunan, N., K. Ritz, D. Crabb, K. Harris, K. J. Wu, J. W. Crawford and I. M. Young. 2001. Quantification of the *in situ* distribution of soil bacteria by large-scale imaging of thin sections of undisturbed soil. FEMS Microbiology Ecology. 37:67-77.

Nunan, N., K. Wu, I. M. Young, J. W. Crawford and K. Ritz. 2002. *In situ* spatial patterns of soil bacterial populations, mapped at multiple scales, in an arable soil. Microbial Ecology. 44:296-305.

Nunan, N., K. Wu, I. M. Young, J. W. Crawford and K. Ritz. 2003. Spatial distribution of bacterial communities and their relationships with the micro-architecture of soil. FEMS Microbiology Ecology. 44:203-215.

Oberrath, R. and K. Bohning-Gaese. 2001. The Signed Mantel test to cope with autocorrelation in comparative analyses. Journal of Applied Statistics. 28:725-736.

Ocio, J. A., P. C. Brookes and D. S. Jenkinson. 1991a. Field incorporation of straw and its effects on soil microbial biomass and soil inorganic-N. Soil Biology and Biochemistry. 23:171-176.

Ocio, J. A., J. Martinez and P. C. Brookes. 1991b. Contribution of straw-derived N to total microbial biomass-N following incorporation of cereal straw to soil. Soil Biology and Biochemistry. 23:655-659.

Odum, E. P. 1983. Basic ecology. Philadelphia, Saunders College Publishing.

Ogram, A. and X. Feng. 1997. Methods of soil microbial community analysis. *In:* Hurst, C. J., G. R. Knudsen, M. J. McInerney, L. D. Stetzenback and M. V. Walter (ed.), Manual of environmental microbiology. Washington, D. C., American Society for Microbiology Press. pp 422-430.

O'Neill, R. V. and A. W. King. 1998. Homage to St. Michael; or, why are there so many books on scale? *In:* Peterson, D. L. and V. T. Parker (ed.), Ecological scale: theory and applications. New York, Columbia University Press. pp 3-15.

Øvreås, L., L. Forney, F. L. Daae and V. Torsvik. 1997. Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Applied and Environmental Microbiology. 63:3367-3373.

Paffetti, D., C. Scotti, S. Gnocchi, S. Fancelli and M. Bazzicalupo. 1996. Genetic diversity of an Italian *Rhizobium meliloti* population from different Medicago sativa varieties. Applied and Environmental Microbiology. 62:2279-2285.

Palmer, M. W. 1990. Spatial scale and patterns of species-environment relationships in hardwood forest in the North Carolina piedmont. Coenoses. 5:79-87.

Pankhurst, C. E., K. Ophel-Keller, B. M. Doube and V. Gupta. 1996. Biodiversity of soil microbial communities in agricultural systems. Biodiversity and Conservation. 5:197-209.

Parkin, T. B., J. L. Starr and J. J. Meisinger. 1987. Influence of sample size on measurement of soil denitrification. Soil Science Society of America Journal. 51:1492-1501.

Parkin, T. B. 1993. Spatial variability of microbial processes in soil - a review. Journal of Environmental Quality. 22:409-417.

Parry, S., P. Renault, C. Chenu and R. Lensi. 1999. Denitrification in pasture and cropped soil clods as affected by pore space structure. Soil Biology and Biochemistry. 31:493-501.

Pedersen, K., J. Arlinger, L. Hallbecky and C. Pettersson. 1996. Diversity and distribution of subterranean bacteria in groundwater in Okla in Gabon, Africa, as determined by 16S rRNA gene sequencing. Molecular Ecology. 5:427-436.

Penner, G. A., A. Bush, R. Wise, W. Kim, L. Domier, K. Kasha, A. Laroche, G. Scoles, S. J. Molnar and G. Fedak. 1993. Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. PCR Methods and Applications. 2:341-345.

Pepper, I. L. and S. D. Pillai. 1994. Detection of specific DNA sequences in environmental samples via polymerase chain reaction. *In:* Weaver, R. and J. S. Angle. Methods of Soil Analysis, Part 2: Microbiological and biochemical properties. Madison, Wisconsin. Soil Science Society of America. pp 707-727.

Pernthaler, J., T. Posch, K. Simek, J. Vrba, R. Amann and R. Psenner. 1997. Contrasting bacterial strategies to coexist with a flagellate predator in an experimental microbial assemblage. Applied and Environmental Microbiology. 63:596-601.

Pettersson, B., K. E. Johansson and M. Uhlen. 1994. Sequence analysis of 16S rRNA from mycoplasmas by direct solid-phase DNA sequencing. Applied and Environmental Microbiology. 60:2456-2461.

Picard, C., C. Ponsonnet, E. Paget, X. Nesme and P. Simonet. 1992. Detection and enumeration of bacteria in soil by direct extraction and polymerase chain reaction. Applied and Environmental Microbiology. 58:2717-2722.

Pielou, E. C. 1966. Shannon's formula as a measure of specific diversity: its use and misuse. The American Naturalist. 100:463-465.

Pielou, E. C. 1969. An introduction to mathematical ecology. New York, Wiley-Interscience.

Pielou, E. C. 1975. Ecological diversity. New York, John Wiley & Sons, Inc.

Pimm, S. L. 1984. The complexity and stability of ecosystems. Nature. 307:321-326.

Pinel-Alloul, B., T. Niyonsenga and P. Legendre. 1995. Spatial and environmental components of freshwater zooplankton structure. Ecoscience. 2:1-19.

Poulsen, L. K., F. S. Lan, C. S. Kristensen, P. Hobolth, S. Molin and K. A. Krogfelt. 1994. Spatial-distribution of *Escherichia-Coli* in the mouse large-intestine inferred from ribosomal-RNA *in-situ* hybridization. Infection and Immunity. 62:5191-5194.

Powell, W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey and A. Rafalski. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular breeding. 2:225-238.

Preston, S., B. S. Griffiths and I. M. Young. 1999. Links between substrate additions, native microbes, and the structural complexity and stability of soils. Soil Biology and Biochemistry. 31:1541-1547.

Putman, R. J. 1994. Community ecology. London, Chapman & Hall Publishers.

Rahel, F. J. 1990. The hierarchical nature of community persistence - a problem of scale. American Naturalist. 136:328-344.

Ramakrishnan, B., T. Lueders, R. Conrad and M. Friedrich. 2000. Effect of soil aggregate size on methanogenesis and archaeal community structure in anoxic rice field soil. FEMS Microbiology Ecology. 32:261-270.

Ranjard, L., F. Poly, J. Combrisson, A. Richaume, F. Gourbiere, J. Thioulouse and S. Nazaret. 2000. Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA). Microbial Ecology. 39:263-272.

Rao, C. R. 1980. Diversity and dissimilarity coefficients: a unified approach. Theoretical Population Biology. 21:24-43.

Rasmussen, L. D. and S. J. Sorensen. 2001. Effects of mercury contamination on the culturable heterotrophic, functional and genetic diversity of the bacterial community in soil. FEMS Microbiology Ecology. 36:1-9.

Riley, M. 1999. Correlates of smallest sizes for microorganisms. *In:* Size limits of very small microorganisms: proceedings of a workshop, National Research Council. pp 21-25.

Ritz, K., B. Boag, C. D. Clegg, A. Gollotte, L. A. Glover, S. J. Grayston, B. S. Griffiths, A. E. McCaig, J. M. McNicol, P. Millard, J. I. Prosser and R. E. Wheatley. 2001. Characterizing spatial variation in microbial community structure in upland grasslands. Abstracts of the 9th International Symposium on Microbial Ecology, Amsterdam, the Netherlands.

Roberts, M. S., J. L. Garland and A. L. Mills. 2003. Microbial astronauts: assembling microbial communities for Advanced Life Support Systems. Microbial Ecology. *In press*.

Robertson, G. P. 1987. Geostatistics in ecology: interpolating with known variance. Ecology 68:744-748.

Robertson, G. P., M. A. Huston, F. C. Evans and J. M. Tiedje. 1988. Spatial variability in a successional plant community: patterns of nitrogen availability. Ecology. 69:1517-1524.

Robertson, G. P., J. R. Crum and B. G. Ellis. 1993. The spatial variability of soil resources following long-term disturbance. Oecologia. 96:451-456.

Robertson, G. P. and K. L. Gross. 1994. Assessing the heterogeneity of belowground resources: quantifying pattern and scale. *In:* Caldwell, M. M. and R. W. Pearcy (ed.), Exploitation of environmental heterogeneity by plants. San Diego, CA. Academic Press. pp 237-253.

Robertson, G. P. and D. W. Freckman. 1995. The spatial distribution of nematode trophic groups across a cultivated ecosystem. Ecology. 76:1425-1432.

Robertson, G. P., K. M. Klingensmith, M. J. Klug, E. A. Paul, J. R. Crum and B. G. Ellis. 1997. Soil resources, microbial activity, and primary production across an agricultural ecosystem. Ecological Applications. 7:158-170.

Ronimus, R. S., L. E. Parker and H. W. Morgan. 1997. The utilization of RAPD-PCR for identifying thermophilic and mesophilic Bacillus species. FEMS Microbiology Letters. 147:75-79.

Rosenzweig, M. L. 1995. Species diversity in space and time. New York, Cambridge University Press.

Rossi, R. E., D. J. Mulla, A. G. Journel and E. H. Franz. 1992. Geostatistical tools for modeling and interpreting ecological spatial dependence. Ecological Monographs. 62:277-314.

Rossi, J.-P. 1996. Statistical tool for soil biology. XI. Autocorrelogram and Mantel test. European Journal of Soil Biology. 32:195-203.

Rothemund, C., R. Amann, S. Klugbauer, W. Manz, C. Bieber, K. H. Schleifer and P. Wilderer. 1996. Microflora of 2,4-dichlorophenoxyacetic acid degrading biofilms on gas permeable membranes. Systematic and Applied Microbiology. 19:608-615.

Saetre, P. 1999. Spatial patterns of ground vegetation, soil microbial biomass and activity in a mixed spruce-birch stand. Ecography. 22:183-192.

Saetre, P. and E. Bååth. 2000. Spatial variation and patterns of soil microbial community structure in a mixed spruce-birch stand. Soil Biology and Biochemistry. 32:909-917.

Salonius, P. O. 1981. Metabolic capabilities of forest soil microbial populations with reduced species diversity. Soil Biology and Biochemistry. 13:1-10.

Sarathchandra, S. U., A. Ghani, G. W. Yeates, G. Burch and N. R. Cox. 2001. Effect of nitrogen and phosphate fertilizers on microbial and nematode diversity in pasture soils. Soil Biology and Biochemistry. 33:953-964.

Savin, M. C., J. H. Gorres, D. A. Neher and J. A. Amador. 2001. Biogeophysical factors influencing soil respiration and mineral nitrogen content in an old field soil. Soil Biology and Biochemistry. 33:429-438.

Scala, D. J. and L. J. Kerkhof. 2000. Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis. Applied and Environmental Microbiology. 66:1980-1986.

Schlesinger, W. H., J. A. Raikes, A. E. Hartley and A. E. Cross. 1996. On the spatial pattern of soil nutrients in desert ecosystems. Ecology. 77:364-374.

Schneider, D. C. 1994. Quantitative ecology - spatial and temporal scaling. San Diego, Academic Press, Inc.

Schneider, D. C. 1998. Applied scaling theory. *In:* Peterson, D. L. and V. T. Parker (ed.), Ecological scale: theory and applications. New York, Columbia University Press. pp 253-269.

Semprini, L., P. V. Roberts, G. D. Hopkins and P. L. McCarty. 1990. Field evaluation of *in-situ* biodegradation of chlorinated ethenes. Part 2, results of biostimulation and biotransformation experiments. Ground Water. 28:715-727.

Seymour, J. R., J. G. Mitchell, L. Pearson and R. L. Waters. 2000. Heterogeneity in bacterioplankton abundance from 4.5 millimetre resolution sampling. Aquatic Microbial Ecology. 22:143-153.

Shannon, C. E. and W. Weaver. 1949. The mathematical theory of communication. Urbana, University of Illinois Press.

Sieburth, J. M. 1984. Protozoan bacteriovory in pelagic marine waters. *In:* Hobbie, J. E. and P. J. L. Williams (ed.), Heterotrophic activity in the sea. New York, Plenum Press. pp 405-444.

Sinclair, J. L. and W. C. Ghiorse. 1987. Distribution of protozoa in subsurface sediments of a pristine groundwater study site in Oklahoma. Applied and Environmental Microbiology. 53:1157-1163.

Sinclair, J. L. and M. Alexander. 1989. Effect of protozoan predation on relative abundance of fast- and slow-growing bacteria. Canadian Journal of Microbiology. 35:578-582.

Smith, D. F. 1986. Small-scale spatial heterogeneity in dissolved nutrient concentration. Limnology and Oceanography. 31:167-171.

Smith, J. L., J. J. Halvorson and H. Bolton. 1994. Spatial relationships of soil microbial biomass and C and N mineralization in a semiarid shrub-steppe ecosystem. Soil Biology and Biochemistry. 26:1151-1159.

Smith, R. L. and J. H. Duff. 1988. Denitrification in a sand and gravel aquifer. Applied and Environmental Microbiology. 54:1071-1078.

Smouse, P. E., J. C. Long and R. R. Sokal. 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Systematic Zoology. 35:627-632.

Sneath, P. H. and R. R. Sokal. 1973. Numerical taxonomy. San Francisco. W. H. Freeman and Company.

Sokal, R. R. 1979. Testing statistical significance of geographic variation patterns. Systematic Zoology. 28:227-232.

Sokal, R. R., J. Bird and B. Riska. 1980. Geographic variation in *Pemphigus populicaulis* (Insecta: Aphididae) in eastern North America. Biological Journal of the Linnean Society. 14:163-200.

Sorensen, P., J. N. Ladd and M. Amato. 1996. Microbial assimilation of ^{14}C of ground and unground plant materials decomposing in a loamy sand and a clay soil. Soil Biology and Biochemistry. 28:1425-1434.

Stackebrandt, E. and F. A. Rainey. 1995. Partial and complete 16S rDNA sequences, their use in generation of 16S rDNA phylogenetic trees and their implications in molecular ecology studies. *In:* Akkermans, A. D. L., J. D. Van Elsas, and F. J. De Bruijn (ed.), Molecular Microbial Ecology Manual. Dordrecht, Netherlands. Kluwer Academic Publishers. pp 3.1.1:1-17.

Stahl, D. 1997. Molecular approaches for the measurement of density, diversity, and phylogeny. *In:* Hurst, C. J., G. R. Knudsen, M. J. McInerney, L. D. Stetzenback and M. V. Walter (ed.), Manual of environmental microbiology. Washington, D. C., American Society for Microbiology Press. pp 102-114.

Stein, A., J. Riley and N. Halberg. 2001. Issues of scale for environmental indicators. Agriculture Ecosystems & Environment. 87:215-232.

Stenger, R., E. Priesack and F. Beese. 2002. Spatial variation of nitrate-N and related soil properties at the plot-scale. Geoderma. 105:259-275.

Stim, K. P., G. R. Drake, S. E. Padgett and D. L. Balkwill. 1990. 16S Ribosomal RNA sequencing analysis of phylogenetic relatedness among aerobic chemoheterotrophic bacteria in deep aquifer sediments. *In:* Fliermans, C. B. and T. C. Hazen (ed.), Microbiology of the deep subsurface. Aiken, South Carolina. WSRC Information Services S2 117.

Stookey, L. L. 1970. Ferrozine - a new specrophotometric reagent for iron. Analytical Chemistry. 42:779-781.

Stoyan, H., H. De-Polli, S. Bohm, G. P. Robertson and E. A. Paul. 2000. Spatial heterogeneity of soil respiration and related properties at the plant scale. Plant and Soil. 222:203-214.

Swift, M. J. 1984. Microbial diversity and decomposer niches. *In:* Klug, M. J. and C. A. Reddy (ed.), Current perspectives in microbial ecology. Washington, D.C., American Society of Microbiology Press. pp 8-16.

Swofford, D. L., G. J. Olsen, P. J. Waddell and D. M. Hillis. 1996. Phylogenetic Inference. *In:* Hillis, D. M., C. Moritz and B. K. Mable (ed.), Molecular Systematics. Sunderland, Massachusetts, Sinauer Associates, Inc. pp 407-514.

Tanaka, H., S. Sawairi and T. Okuda. 1994. Application of the random amplified polymorphic DNA using the polymerase chain reaction for efficient elimination of duplicate strains in microbial screening (Bacteria). The Journal of Antibiotics. 47:194-200.

Tate, R. L. 1997. Soil microbial diversity research: Whither to now? Soil science. 162:605-606.

Teske, A., T. Brinkhoff, G. Muyzer, D. P. Moser, J. Rethmeier and H. W. Jannasch. 2000. Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. Applied and Environmental Microbiology 66:3125-3133.

Theron, J. and T. E. Cloete. 2000. Molecular techniques for determining microbial diversity and community structure in natural environments. Critical Reviews in Microbiology. 26:37-57.

Tilman, D. 1982. Resource competition and community structure. Princeton, N.J., Princeton University Press.

Tilman, D. and J. A. Downing. 1994. Biodiversity and stability in grasslands. Nature. 367:363-365.

Tilman, D. 1996. Biodiversity: Population versus ecosystem stability. Ecology. 77:350-363.

Tilman, D., D. Wedin and J. Knops. 1996. Productivity and sustainability influenced by biodiversity in grassland ecosystems. Nature. 379:718-720.

Tilman, D., J. Knops, D. Wedin, P. Reich, M. Ritchie and E. Siemann. 1997. The influence of functional diversity and composition on ecosystem processes. Science. 277:1300-1302.

Torsvik, V., J. Goksoyr and F. L. Daae. 1990. High diversity in DNA of soil bacteria. Applied and Environmental Microbiology. 56:782-787.

Torsvik, V., K. Salte, R. Sørheim and J. Goksøyr. 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. Applied and Environmental Microbiology. 56:776-781.

Torsvik, V., J. Goksøyr, F. L. Daae, R. Sørheim, J. Michalsen and K. Salte. 1993. Diversity of microbial communities determined by DNA reassociation technique. *In:* Guerreto, R. and C. Pedrórs-Alió (ed.), Trends in Microbial Ecology. Madrid, Spanish Society for Microbiology. pp 375-378.

Torsvik, V., J. Goksøyr, F. L. Daae, R. Sørheim, J. Michalsem and K. Salte. 1994. Use of DNA analysis to determine the diversity of microbial communities. *In:* Ritz, R., J. Kighton and K. E. Giller (ed.), Beyond the Biomass. New York, British Society of Soil Science, Wiley-Sayce Publishers. pp 39-48.

Torsvik, V., R. Sorheim and J. Goksoyr. 1996. Total bacterial diversity in soil and sediments: a review. Journal of Industrial Microbiology and Biotechnology. 17:170-178.

Torsvik, V., F. L. Daae, R. A. Sandaa and L. Øvreås. 1998. Novel techniques for analyzing microbial diversity in natural and perturbed environments. Journal of Biotechnology. 64:53-62.

Torsvik, V., L. Øvreås and T. F. Thingstad. 2002. Prokaryotic diversity - Magnitude, dynamics, and controlling factors. Science. 296:1064-1066.

Torsvik, V. and L. Øvreås. 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology. 5:240-245.

Toyota, K., K. Ritz, S. Kuninaga and M. Kimura. 1999. Impact of fumigation with metam sodium upon soil microbial community structure in two Japanese soils. Soil Science and Plant Nutrition. 45:207-223.

Troussellier, M., P. Lebaron, B. Baleux and P. Got. 1993. Spatial-distribution patterns of heterotrophic bacterial- populations in a coastal ecosystem (Thau Basin, France). Estuarine Coastal and Shelf Science. 36:281-293.

Tulnid, A. and D. C. White. 1990. Use of lipid biomarkers in environmental samples. *In:* Fox, A., S. L. Morgan, L. Lennart, and G. Odham (ed.), Analytical Microbial Methods. New York, Plenum Press. pp 259-274.

Underwood, A. J. and M. G. Chapman. 1998. A method for analyzing spatial scales of variation in composition of assemblages. Oecologia. 117:570-578.

Valentine, D. W., E. A. Holland and D. S. Schimel. 1994. Ecosystem and physiological controls over methane production in northern wetlands. Journal of Geophysical Research-Atmospheres. 99:1563-1571.

Wachinger, G., S. Fiedler, K. Zepp, A. Gattinger, M. Sommer and K. Roth. 2000. Variability of soil methane production on the micro-scale: spatial association with hot spots of organic material and Archaeal populations. Soil Biology and Biochemistry. 32:1121-1130.

Wagner, M., M. Horn and H. Daims. 2003. Fluorescence *in situ* hybridization for the identification and characterization of prokaryotes. Current Opinion in Microbiology. 6:302-309.

Waldrop, M. P., T. C. Balser and M. K. Firestone. 2000. Linking microbial community composition to function in a tropical soil. Soil Biology and Biochemistry. 32:1837-1846.

Wang, G. C.-Y. and Y. Wang. 1997. Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. Applied and Environmental Microbiology. 63:4645-4650.

Wang, Y., Z. S. Zhang and R. Narendrakumar. 1997. The actinomycete *Thermobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. Journal of Bacteriology. 179:3270-3276.

Ward, N., F. A. Rainey, B. M. Goebel and E. Stackebrandt. 1995. Identifying and culturing the 'unculturables': a challenge for microbiologists. *In:* Allsopp, D, R. R. Colwell and D. L. Hawksworth (ed.), Microbial diversity and ecosystem function. Wallingford, CAB International. pp 89-110.

Wardle, D. A. and K. E. Giller. 1996. The quest for a contemporary ecological dimension to soil biology. Soil Biology and Biochemistry. 28:1549-1554.

Wassel, R. A. and A. L. Mills. 1983. Changes in water and sediment bacterial community structure in a lake receiving acid mine drainage. Microbial Ecology. 9:155-169.

Watve, M. G. and R. M. Gangal. 1996. Problems in measuring bacterial diversity and a possible solution. Applied and Environmental Microbiology. 62:4299-4301.

Webster, G., T. M. Embley and J. I. Prosser. 2002. Grassland management regimens reduce small-scale heterogeneity and species diversity of beta-proteobacterial ammonia oxidizer populations. Applied and Environmental Microbiology. 68:20-30.

Webster, R. and F. Butler. 1976. Soil classification and survey studies at Ginninderra. Austrailian Journal of Soil Research. 14:1-24.

Weidner, S., W. Arnold and A. Pühler. 1996. Diversity of uncultured microorganisms associated with Seagrass *Halophila stipulacea* estimated by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Applied and Environmental Microbiology. 62:766-771.

Wenderoth, D. F. and H. H. Reber. 1999. Correlation between structural diversity and catabolic versatility of metal-affected prototrophic bacteria in soil. Soil Biology and Biochemistry. 31:345-352.

White, D. C., G. A. Smith, M. J. Gehron, J. H. Parker, R. H. Findlay, R. F. Martz and H. L. Fredrickson. 1983. The groundwater aquifer microbiota: Biomass, community structure, and nutritional status. Developments in Industrial Microbiology. 24:201-212.

White, D. C. 1995. Chemical ecology: Possible linkage between macro- and microbial ecology. Oikos. 74:177-184.

White, D. C., H. C. Pinkart and D. B. Ringelberg. 1997. Biomass measurements: biochemical approaches. *In:* Hurst, C. J., G. R. Knudsen, M. J. McInerney, L. D. Stetzenback and M. V. Walter (ed.), Manual of environmental microbiology. Washington, D. C., American Society for Microbiology Press. pp 91-101.

Wiens, J. A., J. F. Addicott, T. J. Case and J. Diamond. 1986. The importance of spatial and temporal scale in ecological investigations. *In:* Diamond, J. and T. J. Case (ed.), Community ecology. New York, Harper & Row. pp 145-172.

Wikström, P., A.-C. Andersson and M. Forsman. 1999. Biomonitoring complex microbial communities using random amplified polymorphic DNA and principal component analysis. FEMS Microbiology Ecology. 28:131-139.

Wikström, P., L. Hägglund and M. Forsman. 2000. Structure of a natural microbial community in a nitroaromatic-contaminated groundwater is altered during biodegradation of extrinsic, but not intrinsic substrates. Microbial Ecology. 39:203-210.

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research. 18:6531-6535.

Williams, J. G. K., M. K. Hanafey, J. A. Rafalski and S. V. Tingey. 1993. Genetic analysis using random amplified polymorphic DNA markers. Methods in Enzymology 218: 704-740.

Wilson, M. S., C. Bakermans and E. L. Madsen. 1999. *In situ*, real-time catabolic gene expression: Extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. Applied and Environmental Microbiology. 65:80-87.

Wise, M. G., J. V. McArthur and L. J. Shimkets. 1997. Bacterial diversity of a Carolina Bay as determined by 16S rRNA gene analysis: confirmation of novel taxa. Applied and Environmental Microbiology. 63:1505-1514.

Wollum, II, A. G. and D. K. Cassel. 1984. Spatial variability in *Rhizobium japonicum* in two North Carolina (USA) soils. Soil Science Society of America Journal. 48:1082-1086.

Wright, D. A., K. Killham, L. A. Glover and J. I. Prosser. 1995. Role of pore-size location in determining bacterial-activity during predation by protozoa in soil. Applied and Environmental Microbiology. 61:3537-3543.

Wright, R. T. and B. K. Burnison. 1979. Heterotrophic activity measured with radiolabelled organic substrates. *In:* Costerton, J. W. and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity, and ecology. American Society for Testing and Materials (ASTM STP 695). pp 140-155.

Wu, X. L., K. J. Chin and R. Conrad. 2002. Effect of temperature stress on structure and function of the methanogenic archaeal community in a rice field soil. FEMS Microbiology Ecology. 39:211-218.

Wyland, L. J., L. E. Jackson and K. F. Schulbach. 1995. Soil-plant nitrogen dynamics following incorporation of a mature rye cover crop in a lettuce production system. Journal of Agricultural Science. 124:17-25.

Xia, X., J. Bollinger and A. Ogram. 1995. Molecular genetic analysis of the response of three soil microbial communities to the application of 2,4-D. Molecular Ecology. 4:17-28.

Xing, J., C. Criddle and R. Hickey. 1997. Long-term adaptive shifts in anaerobic community structure in response to a sustained cyclic substrate perturbation. Microbial Ecology. 33:50-58.

Yang, Y.-H., J. Yao, S. Hu and Y. Qi. 2000. Effects of agricultural chemicals on DNA sequence diversity of soil microbial community: a study with RAPD marker. Microbial Ecology. 39:72-79.

Yin, B., D. Crowley, G. Sparovek, W. J. De Melo and J. Borneman. 2000. Bacterial functional redundancy along a soil reclamation gradient. Applied and Environmental Microbiology. 66:4361-4365.

Zabeau, M. and P. Vos. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Office Publication Number EP 0534858 A1.

Zak, J. C., M. R. Willig, D. L. Moorhead and H. G. Wildman. 1994. Functional diversity of microbial communities - a quantitative approach. Soil Biology and Biochemistry. 26:1101-1108.

Zelles, L., A. Palojarvi, E. Kandeler, M. Von Lutzow, K. Winter and Q. Y. Bai. 1997. Changes in soil microbial properties and phospholipid fatty acid fractions after chloroform fumigation. Soil Biology and Biochemistry. 29:1325-1336.

Zheng, D., E. W. Alm, D. A. Stahl and L. Raskin. 1996. Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Applied and Environmental Microbiology. 62:4504-4513.

Zhou, J. and D. K. Thompson. 2002. Challenges in applying microarrays to environmental studies. Current Opinion in Biotechnology. 13:204-207.

Zhou, J. Z., B. C. Xia, D. S. Treves, L. Y. Wu, T. L. Marsh, R. V. O'Neill, A. V. Palumbo and J. M. Tiedje. 2002. Spatial and resource factors influencing high microbial diversity in soil. Applied and Environmental Microbiology. 68:326-334.

Zhou, J. 2003. Microarrays for bacterial detection and microbial community analysis. Current Opinion in Microbiology. 6:288-294.

Zogg, G. P., D. R. Zak, D. B. Ringelberg, N. W. MacDonald, K. S. Pregitzer and D. C. White. 1997. Compositional and functional shifts in microbial communities due to soil warming. Soil Science Society of America Journal. 61:475-481

Zyagintse, D. 1994. Vertical distribution of microbial communities in soils. *In:* Ritz, R., J. Kighton and K. E. Giller (ed.), Beyond the Biomass. New York, British Society of Soil Science, Wiley-Sayce Publishers. pp 29-37.