Chapter 5

THE EFFECTS OF FINE SCALE ENVIRONMENTAL VARIATION ON MICROBIAL COMMUNITY STRUCTURE AND FUNCTION IN AQUATIC ENVIRONMENTS

Kurt A. Smart¹, Hsuanhua L. Smart# and Colin R. Jackson¹*

¹The University of Mississippi, Department of Biology, Shoemaker Hall, MS, USA
#The University of Mississippi, Department of Mathematics, Hume Hall, MS, USA

ABSTRACT

Molecular and biochemical approaches have revealed that microbial communities in natural aquatic environments are more diverse than previously thought. However, few studies have examined how fine scale changes in environmental conditions can alter the structure, diversity, and function of aquatic microorganisms. We performed a number of studies that examined the influence of fine scale changes such as particle size or spatial location on microbial communities in freshwater systems. Bacterial communities associated with different sizes of particles in wetland sediment were characterized using 16S rRNA gene sequencing, which revealed fundamental differences in the structure of communities associated with very fine (0.063-0.25 mm diameter) and fine (0.25-1 mm) particles. Similarly, microbial activity was also influenced by particle size with different activities of lignin and cellulose degrading enzymes associated with different sized particles. Fine (cm) scale spatial variation in microbial activity was also seen on decomposing leaves in a stream, and spatial patterns in cellulose degrading enzymes suggest that the process of organic matter decomposition shows high spatial heterogeneity. Together, these findings demonstrate that aquatic microbial communities vary both structurally and functionally over finer scales than are usually considered in environmental microbiology. This variation should be considered in future surveys of microbial diversity and activity in environmental samples.

* Send correspondence to Dr. Colin R. Jackson.
INTRODUCTION

Interest in microbial community structure and function in aquatic ecosystems has been increasing dramatically. The significance of microorganisms in biogeochemical cycles and ecosystem function has been noted since Lindeman [1] placed the bacterial ‘ooze’ at the center of his trophic cycle, but until the last few decades bacteria were often regarded as a single entity in aquatic environments as studying them in any detail was difficult. Recent developments in molecular and biochemical techniques are helping to overcome that difficulty and are beginning to shed some light on microbial processes at very fine scales [2]. These new methods and techniques are allowing more accurate, detailed descriptions of microbial communities and their activities, and are revealing how diverse these communities are, both in structure and function.

To understand how microorganisms interact with each other and with their environment, it is important to consider the scale at which the interactions occur. Microorganisms respond to localized, fine-scale changes in their environment, both spatially and temporally, and it is important that these responses and interactions be understood more thoroughly [3]. On a hierarchal scale, where organizational levels are created from lower levels but also act to create higher levels of organization, it is important to consider multiple levels as the relationships between levels are what provide the insights into the function of the system [4]. Lower levels of hierarchal organization (i.e. fine scales) are highly variable and operate at high rates, but those rates can be averaged out when looking at the system from higher levels [5]. By overlooking processes and patterns at fine scales, any variability that may be present is missed, making it impossible to know what is actually occurring to create the larger scale system and its function. Looking at patchiness in planktonic organisms, Krembs et al. [6] argue that concentration dependent rate processes may be underestimated by failing to take into account fine-scale spatial variability. Knowledge of such fine-scale processes are critical in understanding larger scale phenomenon, such as ecosystem or global level cycles, as fine-scale interactions and activities of microorganisms combine together to form these larger scale processes. Until recently, the ability to observe and measure fine-scale microbial processes was very limited.

Early studies of environmental microbiology relied upon culture-based methods for describing microbial communities. However, the vast majority of prokaryotic species could not be cultured, and most still have yet to be cultured using current culturing techniques. Culture-based methods greatly underestimate the diversity of natural microbial communities, and may select for organisms which either do not make up a significant portion of the community or do not contribute a great deal to the activity of the community, providing a skewed representation of what is actually present. Rapid advances in molecular and biochemical methods have allowed microbial communities to be described without the bias involved in culturing techniques [2]. They have also become increasingly sensitive, enhancing the ability for measurement on finer and finer scales. The application of the polymerase chain reaction (PCR) has been the most significant development for dealing with environmental microorganisms, allowing replication of minute amounts of environmental DNA into quantities large enough to be analyzed. The use of fluorescently labeled compounds has also greatly benefited environmental microbiology, detecting organisms and measuring a wide range of enzymatic or metabolic processes at very high sensitivities.
Fluorescent in-situ hybridization (FISH) combined with confocal laser microscopy has enabled direct observations of microbial community structure [7]. Along with molecular tools and advances in microscopy, the development of new biochemical assays and instruments have proved helpful in measuring fine-scale activities and function [8]. Microsensors have also been widely implemented in determining microenvironmental conditions and processes, measuring chemical concentrations and gradients over very fine scales [9]. The use of such methods and several more are discussed in this chapter, primarily focusing on the effects of fine-scale environmental variation on aquatic microbial communities. We follow the discussion by presenting two examples of studies that we performed examining fine scale patterns in the structure and function of microbial communities in aquatic environments.

Microorganisms do not show homogeneous distribution patterns in any aquatic environment. Microbial species composition and abundance in lakes are known to vary with depth, depending upon physical (e.g. light availability, temperature), chemical (e.g. O₂, CO₂), and biological (e.g. symbiotic or predator-prey relationships) characteristics [10]. They are also known to vary between littoral and pelagic habitats, and seasonally [10]. In lotic systems the distribution varies more with horizontal rather than vertical profiles because of differences in hydrological conditions [11]. However, our knowledge of that variability is based upon observations at spatial scales of meters or tens of meters, and temporal scales of weeks to months. Microorganisms have more recently been shown to be heterogeneously distributed throughout the water column, on submerged surfaces, and in aquatic sediments over much finer spatial and temporal scales.

**Fine Scale Variation in Microbial Communities in the Water Column of Aquatic Environments**

Microorganisms are found to be very heterogeneously distributed in the water column of aquatic environments. Bacterioplankton abundance has been shown to vary in pelagic marine environments on a scale of several millimeters [12], and at even finer scales bacteria have been shown to form aggregates or associations on or near suspended organic particles [13] and phytoplankton [14], or with other microorganisms [15]. Microbial communities have even been observed to be patchily distributed on the surfaces of particulate matter, demonstrating how small the microenvironments that they inhabit are [16]. There appear to be several reasons for this non-uniform distribution.

The most obvious reason is the availability of nutrients. Live phytoplankton release photosynthetic byproducts that attract high concentrations of bacteria [14]. Phytoplankton are not distributed evenly throughout the water column, and may be more heterogeneously distributed than previously thought, forming thin layers or concentrated sheets that may be only centimeters thick [17]. These concentrated layers of phytoplankton are also highly concentrated with bacteria. Particulate organic matter (POM) is also a prime source of nutrients and energy, and is the remains of larger organic matter which has been partially degraded by bacteria and fungi. In comparison to the surrounding water column, POM is rich in nutrients, and microorganisms aggregate around POM, increasing overall spatial heterogeneity [18]. Over fine scales the distribution of detrital particles is not uniform, which further increases the heterogeneous distribution of microorganisms [18]. Detrital particles
vary greatly in size, shape, and nutrient composition, but in all cases it is a much more plentiful source of nutrients and carbon for bacteria and fungi than scavenging in the surrounding water.

The majority of particulate matter-microbial community research has focused on particulate matter towards the larger end of the size scale. These large particles, or macroaggregates, dubbed ‘marine snow’, ‘lake snow’, and more recently ‘river snow’, were the first to be studied in detail as they are easily visible with the naked eye (typically ≥0.5 mm in diameter). However, macroaggregates are formed from finer particles which are bound together with transparent exopolymer particles produced by algae, diatoms, and bacteria [19]. These aggregates are composed of a wide variety of smaller particles, such as diatom frustules, dead phytoplankton cells, fecal matter, or decomposing plant matter, and are highly variable in both nutrient content and availability. Nutrients in POM are not homogenously distributed, and not all particles are equal in nutritional quality or availability; fine-scale heterogeneity of attached microorganisms may therefore be increased by the surfaces of the particles they are attached to.

A variety of forces, both physical and biological, act to create very steep physical and chemical gradients associated with POM. Respiration of heterotrophic bacteria colonizing both the interior and surface of particles can create an oxygen gradient [20] and the diffusion of gases and dissolved nutrients is constrained within the inner core of aggregates [21]. Smith et al. [22] measured the activity of a wide range of hydrolytic enzymes on individual aggregates of marine snow collected off the California coast and found high variability in all of the enzymes tested, suggesting their are major differences between particles. The surface area of POM is more variable than is normally considered, which may explain some of the variability in activity and chemical and physical gradients. Over very fine scales the surface of detritus can be very fractal in nature, which would mean that the microbial communities are not spread evenly across a perfect sphere as is often assumed [23]. Interstitial spaces may also be highly variable between particles [24]. These gradients may lead to differences in community structure as they create a wide range of microenvironments with dramatically different physical conditions.

Not all of the microenvironments occur on or in the particle itself, however, such as the formation of nutrient plumes released from sinking POM. On the surface of POM, heterotrophic bacteria and fungi attached to the surface produce extracellular enzymes, hydrolyzing the POM and releasing nutrients which are then free to be taken up by the colonizing microbes. Not all of the nutrients released by this enzymatic degradation are taken up by the colonizing microorganisms, and some may leak into the surrounding water column [13]. As a particle sinks the fluid flow past the particle creates a trail, or plume, of released nutrients in the wake of the particle. These plumes have been observed to be several meters in length and remain intact for several minutes [25]. Plumes of this nature attract chemotactic bacteria, zooplankton, and protists which are thought to be well-adapted to life unattached to particles and may also play a role in attracting new inhabitants to particles [25].

All of the particles examined to date have been found to support highly abundant and diverse microbial communities, with the species composition of suspended particles being shown to be different from that of free-living microbial populations. In a study of the Columbia River estuary, Crump et al. [26] compared the 16S rRNA gene sequences of aggregate-associated bacteria with those of free-living bacteria and observed major differences in freshwater, saltwater, and brackish environments. DeLong et al. [27] compared
the 16S rRNA gene sequences of aggregate-associated bacteria with those of free-living bacteria in marine waters and found they were also very distinct: aggregate-associated species were typically members of the Cytophagales, Planctomycetes, and gamma-Proteobacteria, while the free-living species were all alpha-Proteobacteria. In a similar analysis in Lake Constance, alpha-Proteobacteria were found to be the dominant members of the macroaggregate-associated community along with the beta-Proteobacteria, although alpha-Proteobacteria were not detected on finer microaggregates [28]. This suggests that bacterial communities may be adapted to the microenvironments of particulate matter and possibly to specific niches within those microenvironments. ‘River snow’ also appears to have different aggregate-associated microbial communities [16] and the structure of microbial communities appears to differ between these environments, but the reasons for this are still unknown. We describe differences in bacterial community structure associated with different sizes of particles later in this chapter.

As well as supporting diverse communities of microorganisms, detrital particles are also described as ‘hot-spots’ of microbial activity, supporting microbial populations several orders of magnitude greater than the surrounding environment, with larger, more active individual cells [29]. Growth rates are much greater for attached bacteria because of their elevated activity and the greater availability of nutrients [30]. Upon attaching to surfaces, planktonic bacteria up-regulate their activity and increase production of extracellular enzymes; enzymatic activity of attached bacteria can be orders of magnitude greater than planktonic bacteria [31]. Up-regulation occurs very suddenly, within minutes, and leads to rapid decomposition of the detritus and remineralization of nutrients [25]. There is a very dynamic structure to the fine-scale formation and degradation of macroaggregates, which has significance at larger scales. Macroaggregates are degraded by highly active bacteria and smaller particles are released; the degradation of aggregates effect the rate at which they sink, with larger particles sinking at a greater rate [32]. As particles are degraded and reformed rather rapidly, it may reduce the overall sinking rate and allow more time for microbial remineralization of nutrients, which has major implications for carbon cycling and sedimentation in aquatic environments.

Microorganisms are closely associated on POM and it is likely that competition for resources is high. There is some evidence that suggests microbial communities are functioning as consortia with interspecific interactions that enhance the ability of other microorganisms to colonize the substrate and take up nutrients at a greater rate, as Grossart et al. [33] found that the presence of certain bacterial species on aggregates enhanced the ability of other species to colonize the substrate while reducing the ability of others. It is also likely there are fine spatial and temporal scale successional patterns occurring in these microenvironments [34]. Very little is known about competition, succession, or mutualistic relationships on aggregates, but it seems likely considering the interactions in other closely associated microbial communities, such as biofilms.

A possible advantage to the patchy distribution of microorganisms may be defense against predation. Predation by protists can significantly control bacterial populations, and grazing pressure from protists has been shown to alter the dominant cell morphology within bacterial communities [35]. Other species may form large colonies to avoid predation [15]. Residing on large particles may provide similar benefits, although other studies have found that protists are also closely associated with aggregates and that grazing pressure on aggregates is quite high [13]. Higher bacterial growth rates on POM may be able to overcome
grazing effects, or may be a response to them. It is also possible that particle-associated bacterial communities may form biofilms, producing extracellular polymeric substances (EPS) which may offer some grazing protection. More attention is being given to understanding such interactions, but it is highly speculative and too early to draw any general conclusions.

**FINE SCALE VARIATION IN BIOFILMS**

Microorganisms colonize every available submerged surface in aquatic environments, and attached microorganisms are often present in much higher numbers than microorganisms unattached to surfaces [36]. Microorganisms inhabiting surfaces form densely packed, complex, interactive communities (biofilms) composed of living cells and both organic and inorganic matter heterogeneously distributed in both horizontal and vertical directions and encased in EPS. Biofilms provide an advantage for microorganisms in stressful environments, such as those exposed to extreme temperatures or pH, and almost function as multicellular organisms [37]. Very fine-scale interactions and dynamics, both temporal and spatial, occur within biofilms, between biofilms and the colonized substratum, and between biofilms and the surrounding environment, which exhibit a wide array of functions and metabolic processes and which play a significant role in nutrient and energy cycling in aquatic ecosystems [38].

Bacteria that come into contact with and attach to surfaces form microcolonies which spread both across the surface and upwards above the surface in a three-dimensional manner. This colonization and growth leads to spatial heterogeneity of biofilms as microcolonies approach each other and as microenvironments are formed in both the horizontal and vertical planes. Across the substratum heterogeneity in biofilm structure is caused by both the presence of other inhabitants and by the surface to which the biofilm is attached. Surfaces vary greatly in chemical and physical characteristics, and microbial communities respond to those differences. Jones *et al.* [39] found that the microbial communities on different artificial substrata in an estuary were similar in species composition during the early stages of biofilm formation but were different after a week of incubation. Their results suggest that attachment is at first random, but that characteristics of the substratum favor certain microbial inhabitants over others. At very fine scales surfaces can be highly heterogeneous and fractal in nature, which affects not only the surface area available for colonization but also environmental conditions, such as flow, which can affect pH, nutrient availability, waste removal, and oxygen availability, among others [40, 41]. Fine-scale surface characteristics can have large-scale impacts, such as significant alterations in microbial productivity and respiration in stream ecosystems [42].

Other forces also act to create heterogeneity in both microbial community structure and activity within biofilms. Communication between microbial populations is a dominant factor, responsible for coordinating the activity of the tightly connected communities, as biofilms contain structures not seen in other microbial communities and are very detailed at fine scales [43]. Channels are also formed throughout the biofilm, allowing water to flow through the film, transporting nutrients and wastes. The flow is often less than that at the surface of the biofilm and has been observed to flow both with and against the surface current [44],
demonstrating the differences in physical forces acting upon organisms within biofilms compared to the surface.

A large portion of the biofilm may consist of inactive or dormant cells that are not contributing to the activity measured at larger scales. Francoeur et al. [45] demonstrated this heterogeneity using fluorescently-labeled substrate to detect extracellular protease activity, observing a few highly active cells surrounded by a void of inactive, or ‘dead’, cells. Analysis of a biofilm in drinking water that received an addition of several substrates demonstrated similar spatial heterogeneity in microbial activity [46].

As mentioned previously, communication between microbial populations in biofilms is an important factor that operates at very fine spatial and temporal scales. Chemical signals within biofilm matrices are more concentrated than signals outside of biofilms, likely because of decreased flow rates in biofilms and the high density of extracellular polymeric substances that encapsulate them, reducing the diffusion rate of the signal and localizing its effects [47]. The hydrophobic properties of many of the signal molecules may also allow the signal to remain localized [43]. Other gradients within the biofilm, such as pH gradients, also restrict the dispersion of signals by affecting the degradation rate of the chemical [43]. Restricted diffusion of signals in biofilms is another example of an extremely fine-scale process that could have a tremendous impact on larger scale functions.

In addition to the patterns already noted regarding heterogeneity across surfaces, the vertical dimension of biofilms also creates diverse microenvironments as biofilms are often several hundred micrometers thick and support a wide range of metabolic activities [48]. Diffusion of gases or nutrients through the biofilm matrix is often restricted, and microsensor measurements show that anoxic conditions can occur within 30 micrometers of the surface even if the surface is saturated with oxygen [49]. At different levels within the vertical profile of biofilms there are distinct functional processes occurring and often specific community compositions. Even in single-species biofilms grown under laboratory conditions there can be differentiation of cells and a non-uniform distribution of function [50]. Biofilms contain niches and microenvironments which allow important processes to occur that would otherwise not be possible in the particular environment. Okabe et al. [51] found that sulfate-reducing bacteria were dominant at the oxic-anoxic chemocline in activated sludge biofilms and formed a thin, highly active sulfate-reducing layer in the film. Microenvironments within biofilms enable sulfate-reducing bacteria to be active in an otherwise aerobic environment [52], and other metabolic processes can show similar patterns in biofilms [53, 54].

Protozoan grazing of biofilms also affects the structure and function of biofilm bacterial communities and plays a major role in influencing fine-scale interactions within biofilms. Biofilms might be thought to offer protection against predation by protists, but protozoan grazing can dramatically affect biofilm population dynamics [55]. Grazing has been shown to increase heterogeneity of biofilms as protists preferentially graze on small patches in the biofilm, significantly reducing the local microbial populations [56]. Protists also appear to target areas that others have already grazed, emphasizing local grazing patterns [56]. The cause of this selective grazing is not yet known, but it is likely to have an effect on larger-scale processes.

Temporal scales are also important to consider, as microorganisms present in younger, newly developing biofilms, appear to be much more active than those in mature biofilms [46]. Conditioning of the substratum and the subsequent attachment of microbes occur at very fine temporal scales and growth and spread of colonies over the surface is rapid, with surfaces
introduced to aquatic environments being densely covered in a few days. Over longer time intervals biofilm communities undergo successional changes as the biofilm develops and matures [57]. These changes in community composition are a result of fine-scale interactions and changes in microenvironmental conditions which enable a more diverse set of metabolic activities [58]. Microbial inhabitants of biofilms are closely linked to each other, often forming a consortium of interacting organisms, benefiting one another by co-metabolizing substrates or metabolizing each others waste products. They are very intricately detailed communities which appear to function almost as multicellular organisms.

**FINE SCALE VARIATION IN MICROBIAL COMMUNITIES IN SEDIMENTS**

Aquatic sediments share many of the characteristics of biofilms and suspended POM, and fine-scale heterogeneity of microbial community structure and function are similar. Sediments consist of a wide range of particles that are diverse in both physical and chemical characteristics, varying greatly in size, shape, density, organic and inorganic nutrient composition, and surface features [18]. The environment in which the sediment particles are located are also highly diverse and significantly impact the structure and activity of the microbial biota inhabiting them; river and stream sediments are markedly different than their lake and ocean counterparts, and tidal sediments have their own distinct properties due to the very different physical forces acting upon them [59 - 61]. River and stream sediments are constantly changing as sediments are pushed downstream and resuspended from the benthos. Lake sediments fluctuate because of suspended particles settling out, and tidal sediments fluctuate in response to tidal currents at fine temporal scales.

Sediments are similar to biofilms in regard to their vertical profiles, where different processes occur at different depths and where microenvironmental variations support a diverse range of activities. The inability of nutrients and gases to diffuse evenly through sediments creates layers which are significantly different in composition of nutrients and energy [62]. In lotic systems, turbulent flow that disrupts sediments can greatly increase bacterial density and activity on fine-scales, especially at the sediment surface [63]. Fine-scale fluctuations in pore size and sediment topography also lead to highly diverse patterns in biogeochemical cycles at fine temporal (seconds) and spatial (cm) scales, increasing or decreasing the flow of water through sediments and altering the physical and chemical microenvironment [64].

Dynamics on the surface of particles in sediments are similar to the dynamics on suspended particles, with differences in activity having been measured between different size fractions of particles in sediments in a wide range of aquatic habitats. Finer particles in wetland and river sediments have greater bacterial biomass and productivity compared with coarser particles [65]. Particle size also influences extracellular enzyme activity and bacterial community structure, as we describe later in this chapter.

Uneven distribution of nutrients and energy also results in fine scale heterogeneity in sediments. Particulate matter is not distributed homogeneously in sediments and is not distributed evenly over time. In aquatic systems organic matter is distributed in patches, as in the case of allochthonous plant matter [66] or decaying fish and plankton carcasses [67].
Uneven distribution of organic nutrients in wetland sediments has been shown to be correlated with spatial heterogeneity in methane production [68], suggesting spatial patterns in methanogen communities. Fine-scale spatial heterogeneity may also arise due to metal contamination, as pollutants such as lead and mercury are often patchily distributed [69]. A study of heavy metal contaminated soils revealed that the composition and activity of microbial communities varied dramatically over fine scales, with the potential metabolic activity varying up to 10,000 fold over a single centimeter [70].

Invertebrate burrowing can also increase the heterogeneity of microenvironments in aquatic sediments, creating oxic conditions in layers of sediment that would otherwise be anaerobic [71]. Invertebrates, fish, and other macrofauna are also responsible for disturbing sediments and altering microenvironments, altering fine-scale distributions of nutrients and microorganisms while feeding or traveling along the benthos [72]. Variability at fine scales may also arise because of aquatic plants and their root structure, with bacterial populations in the rhizosphere differing from those in the surrounding sediment [73]. Fine-scale interactions are common within the rhizosphere as a variety of factors influence nutrient availability and environmental conditions over small areas [74].

**THE EFFECTS OF PARTICLE SIZE ON BACTERIAL COMMUNITY STRUCTURE AND ACTIVITY IN WETLAND SEDIMENTS**

As stated previously, particle size influences bacterial biomass and productivity in aquatic sediments [65]. At relatively coarse scales, sediment particle size also influences the structure of the associated microbial community. Particles in aquatic environments can be designated as either coarse particulate organic matter (CPOM, >1 mm diameter) or fine particulate organic matter (FPOM, <1 mm diameter) based upon size [75], and there are differences in the microorganisms associated with these classes of particles. Fungi are thought to dominate the decomposition of CPOM, whereas the smaller size of FPOM limits the growth of fungal hyphae and bacteria tend to be more abundant [76]. However, fewer studies have examined how bacterial community structure changes with decreasing size of FPOM, i.e. how fine scale changes in particle size might influence the attached bacterial community. While FPOM refers to all particles below 1 mm in diameter, it essentially consists of particles that are the remains of decomposing CPOM, and smaller (generally <100-200 μm diameter) particles that may come from a variety of sources, such as the precipitation and flocculation of dissolved material [77]. Given these differences, it seems reasonable to hypothesize that there may be differences in the bacterial communities associated with different sizes of FPOM, and basic DNA-DNA dot blot hybridizations have suggested that this is indeed the case [78]. The DNA-DNA hybridization technique does not directly assess community structure or diversity, however, and interpretation of patterns can be problematic [79]. Thus, we used 16S rRNA sequencing techniques to investigate the structure and diversity of the bacterial community present on different sizes of particles in wetland sediments. A companion study examined microbial extracellular enzyme activity associated with the different sizes of particles.

Sediment samples were collected from a cypress-tupelo swamp in southeastern Louisiana, USA, and sieved into two size ranges of particles (38-250 and 250-1000 μm
Diameter). DNA was extracted from each size range of particles using a modified version of a protocol originally designed for soil [80] but also used for geothermal spring sediment [81]. Residual humic contamination was subsequently removed from extractions using Sepharose 4B spin columns [82]. DNA extracted from both sizes of particles was used as the template in PCR amplifications [81] using primers specific for Domain Bacteria. These primers (Bac8f and Univ1492r) amplify positions 8-1492 of the 16S rRNA gene in Bacteria [83]. PCR products were purified, cloned into artificial plasmid vectors, and clone libraries (one for each size of particles) generated following procedures described previously [84]. The inserts from the first 96 clones obtained in each library were amplified and digested with three separate restriction enzymes (EcoRI, RsaI, HaeIII; 2 h digest at 37°C). After each digest the resulting restriction patterns were visualized on agarose gels. Clones in the same library that showed the same restriction patterns for all three restriction enzymes were assumed to have similar 16S rRNA genes inserted and were grouped together as a single phylotype [81, 84].

The frequency of each phylotype in each clone library was used to estimate the total bacterial richness present on particles of each size range using the non-parametric abundance-based estimator (S\text{Chao1}) of Chao [85]. Calculations were performed using a web interface [86]. For each dominant phylotype, a portion of the 16S rRNA gene insert in one representative clone was sequenced, using the original Bac8f primer. This sequencing protocol yielded approximately 600 bp of sequence information, corresponding to roughly the 50-650 bp region of the 16S rRNA gene. The partial 16S rRNA sequences obtained were compared to those in GenBank to identify closest relatives and subsequently imported into the ARB software package [87]. Sequences were aligned automatically (FastAligner) and alignments checked manually. Because the partial 16S rRNA gene sequences obtained in this study represented a number of diverse lineages, novel phylogenetic trees were not constructed as this might produce erroneous results compared to existing relationships for major bacterial taxa. Rather, ARB’s “Quick add by parsimony” function was used to incorporate the sequences into existing phylogenetic trees in the ARB database. 16S rRNA sequences in the existing trees that were unrelated to those obtained in this study were subsequently removed without altering tree topology, so that phylogenetic trees showing only the lineages specific to this study were obtained. This treeing procedure has been used previously to minimize distortions to established tree topologies that might arise from a phylogenetic analysis of short, highly divergent sequences [84].

When S\text{Chao1} was calculated by repeated random sampling from each library, estimates of the total bacterial phylotype richness in the original community were 177 phylotypes associated with larger particles and 288 phylotypes associated with the finer particles. In each case, S\text{Chao1} began to approach a stable asymptotic value although this was not pronounced for the clone library derived from the finer sediment particles (Figure 1). This suggests that while the estimated richness for the community associated with primary particles is a reasonable approximation, the value of S\text{Chao1} determined for the community associated with the finer, secondary particles is probably an underestimate. Thus, not only does the structure of the bacterial community differ between sediment particles of different size ranges, but overall community properties such as species diversity appear to differ also, with finer particles harboring substantially more diverse bacterial communities. This supports previous observations of increased bacterial community heterogeneity with decreasing FPOM particle size in aquatic sediments, and may be the result of increasing surface area/volume ratios on finer particles, which would result in greater relative habitat area and more available
ecological niches [78]. The greater chemical complexity of secondary particles could also account for increased bacterial diversity on those particles.

![Graph showing predicted species richness vs library sample size](image)

Figure 1. Predicted species (phytotype) richness as determined from analyses of 16S rRNA clone libraries generated from bacterial communities associated with two different sizes of particles (A: 250-1000 μm diameter; B: 38-250 μm diameter) in wetland sediments. Finer particles (B) harbor substantially more diverse bacterial communities than coarser particles (A).

Most 16S rRNA sequences showed low (<97 %) similarity to those already in the GenBank/EMBL/DDBJ databases. The majority of phylotypes occurred just once in each library, and repeated phylotypes were relatively rare. Across both sizes of particles, the sequenced phylotypes represented thirteen major bacterial lineages as well as a number of other taxa that have only been described from molecular studies. Sequences affiliated with the delta- and beta-Proteobacteria were the most abundant sequences found in the clone libraries for both sizes of particles, although the delta-Proteobacteria were more dominant in the library generated from 250-1000 μm particles (Figure 2). Both of these groups, along with the alpha-Proteobacteria are typically dominant in freshwater sediment communities [88]. Sequences affiliated with the alpha-Proteobacteria were abundant in the clone library derived from the larger particles but were comparatively rare in the finer particle library (Figure 2). Similar differences between the two clone libraries were also seen for the Chlorobi (green sulfur bacteria), Nitrospira, and Spirochaetes, the latter being undetected in the clone library derived from 38-250 μm particles. In contrast, the library derived from these finer particles contained more 16S rRNA sequences affiliated with the Verrucomicrobia, Chloroflexi (green
non-sulfur bacteria), and various uncultured bacterial lineages (Figure 2). Thus, the 16S rRNA sequences derived from the different size ranges of particles showed appreciable differences in their phylogenetic affiliation at broad taxonomic levels.

Figure 2. Frequency of major bacterial lineages in 16S rRNA clone libraries generated from PCR-amplified DNA extracted from two different sizes of fine particles (0.25-1 mm diameter and 0.038-0.25 mm diameter) in wetland sediments. Representative of some bacterial lineages were found associated with both sizes of particles, but other groups are specific to just one type of particle, suggesting variation in bacterial community structure at fine scales.

The identity of the dominant phylotypes in each clone library also differed substantially. 16S rRNA sequences affiliated with members of the Bacteroides and the Methylococcales (gamma-Proteobacteria), a group of methanotrophic Bacteria, were the most abundant individual phylotypes in the clone library derived from larger particles, and these phylotypes each accounted for 5% of the clones sequenced. Other specific phylotypes that were abundant in this clone library were affiliated with the Rhodocyclales (beta-Proteobacteria), Pseudomonadales (gamma-Proteobacteria), and Myxococcales (delta-Proteobacteria). None of these orders of Bacteria were represented by abundant phylotypes in the clone library derived from finer particles. As might be expected from its higher diversity, the distribution of cloned sequences in this library was more even, with no individual phylotypes accounting for more than 3% of the community composition. Thus, as well as the two clone libraries differing in overall phylotype diversity and in general phylogenetic composition, they also differed in the specific groups of Bacteria that were most abundant within each.

Only one phylotype was found in both libraries. This is despite sequences being grouped into phylotypes by a fairly liberal 97% sequence similarity criterion, which would have led to increased overlap. This phylotype was affiliated with the Nitrosomonadales (beta-
Proteobacteria) and was represented by just a single clone sampled in each library. Other than that phylotype, the two libraries contained no overlapping 16S rRNA genes, at least as apparent from sampling 96 clones within each. While it’s possible that this may represent inadequate sampling, it strongly suggests that the different size ranges of particles harbor very different bacterial communities.

Differences between bacterioplankton and particle- or sediment-associated bacterial communities have been previously reported for aquatic environments [27, 89], but the influence of particle size on aquatic bacterial community structure or function has rarely been specifically addressed. This is in contrast to terrestrial ecosystems where soil particle size distributions (e.g. sand, silt, and clay) and their associations with organic matter are known to result in spatially heterogeneous environments [90]. This heterogeneity can result in differences in the bacterial populations associated with the different soil particle size fractions, and the influence of particle size on the structure of the bacterial community can be greater than that of larger scale treatments [91]. The results from this study clearly show differences in the bacterial phylotypes present in 16S rRNA clone libraries derived from DNA associated with 250-1000 μm and 38-250 μm diameter FPOM in wetland sediments. Sediment was separated into the two different size ranges by a wet sieving procedure, and because this involves washing the sediment with running water, unattached cells are likely to be removed. Thus, the differences in the bacterial phylotypes present in each size range are likely to reflect actual differences in the microbial communities attached to these types of particles.

Functional differences in the microbial communities associated with different sizes of sediment particles were examined by assaying microbial extracellular enzyme activity. Enzymes such as cellobiohydrolase, beta-glucosidase, and beta-xylosidase are involved in the microbial degradation of lignocellulose and the activity of these enzymes has been linked to rates of POM decomposition [8, 92]. Sediment was collected from the same wetland site and sieved into five different sizes of particles of diameters 1000-2000 μm, 500-1000 μm, 250-500 μm, 125-250 μm, and 63-125 μm. Material in each size range was assayed for the activity of four extracellular enzymes (beta-glucosidase, cellobiohydrolase, beta-xylosidase, and beta-n-acetylglucosaminidase) using artificial p-nitrophenol linked substrates [84, 92]. Absorbance of the resulting end product was determined at 410 nm and converted to micromoles of substrate consumed using standard values [84].

Activity of all four enzymes was correlated (R values > 0.90 for each pair wise comparison) and declined on particles below 500 μm (Figure 3). This decline was consistent as particle size was further reduced, with the lowest activity of all enzymes being found associated with particles collected on the 63 μm sieve. The decline in activity on particles finer than 500 μm strongly suggests functional differences in the attached microbial community, and supports the differences in bacterial community structure reported above. Given that extracellular enzyme activity is related to rates of organic matter decomposition and nutrient cycling, these fine scale differences in microbial activity with particle size suggest that rates of lignocellulose degradation and other aspects of ecosystem function also differ over fine spatial scales.
Variation in microbial enzyme activity and community structure with different sizes of fine particulate organic matter is one example of how fine scale environmental variation affects aquatic microbial communities. A more direct measurement of spatial variation in microbial activity could be made by examining distribution patterns of enzyme activity across the surface of larger particles, such as decomposing leaves in aquatic systems. The surface of living leaves, or phyllosphere, supports variable microbial populations of bacteria and fungi and colonization of the phyllosphere is random and patchy [93]. However, no studies have looked at spatial variation in microbial activity on decomposing leaf-litter at fine scales. In this study we examined the fine-scale variation in microbial extracellular enzyme activity across the surface of decomposing leaves in a small stream. As we stated previously in this chapter, the activities of lignocellulose-degrading enzymes are a reliable measure of rates of cellulose and lignin decomposition, as leaf-litter is composed primarily of lignin and cellulose, both of which require enzymatic degradation to be metabolized [8]. Here we measure the activities of two extracellular enzymes involved in cellulose decomposition (beta-glucosidase and cellobiohydrolase), and a combination of peroxidase and phenol oxidase activity as a broad measurement of enzymes involved in lignin decomposition.
Freshly fallen leaves of sycamore (*Platanus occidentalis*), northern red oak (*Quercus rubra*), scarlet oak (*Quercus coccinea*), and wild black cherry (*Prunus serotina*) were collected from Cypress Creek, a third order stream which flows through Holly Springs National Forest in northern Mississippi. Leaves were collected in the early winter, the period of greatest leaf fall at this site. Five replicate leaves of each species were collected from the surface of the streambed and refrigerated in water collected from the site until processing (1-3 days). On each day of processing, random leaves were chosen for assays of extracellular enzyme activity. Small circular discs (5 mm diameter) were bored at different points across each leaf (typically 15-35 points per leaf) and each disc was subsequently assayed for the activity of one microbial enzyme. Digital images of the remaining leaf (minus discs) were taken to identify the location of sample points for use in the subsequent spatial analysis.

Discs were assayed for enzymes using chromogenic artificial substrates. Beta-glucosidase and cellobiohydrolase activities were determined using the same p-nitrophenol linked substrates as for the wetland sediments study. Leaf discs were incubated with 300 μl substrate at room temperature for 2-4 hours, whereupon the reaction mixture was diluted with NaOH as described previously [84, 92]. Absorbance of the end product was determined at 405 nm [94] and converted to activity on an areal basis of leaf surface (i.e. activity as μmoles of substrate consumed per hour per area). Activities of phenol oxidase and peroxidase were combined into one measurement of overall oxidative enzyme activity using the artificial substrate L-3,4-dihydroxyphenylalanine (L-DOPA) and 0.015% hydrogen peroxide. Leaf discs were incubated in 300 μl of 5 mM L-DOPA with hydrogen peroxide for 2-4 hours and the absorbance of the diluted reaction mixture determined at 450 nm [95]. As with beta-glucosidase and cellobiohydrolase, oxidative enzyme activity was expressed as μmoles of substrate consumed per hour per area of leaf surface for each disc.

Activities of the microbial enzymes on the different discs taken from each leaf were used to calculate mean and standard deviation of activity for each enzyme and leaf species. These values were used to calculate the coefficient of variation (CV), a basic measurement of spatial variability [96, 97]. Spatial patterns in enzyme activity across the leaf surface were visualized by interpolation using the geostatistical software package SADA v.4.1.50 (Spatial Analysis and Decision Assistance, University of Tennessee).

In general, beta-glucosidase showed the highest activity of the three enzymes examined and cellobiohydrolase activity the lowest, regardless of leaf species (Figure 4). Oxidative enzyme activity and beta-glucosidase activity were similar on cherry and scarlet oak leaves (median oxidative activities of 0.04 and 0.05 μmol/h/cm², respectively, and median beta-glucosidase activities of 0.13 and 0.10 μmol/h/cm², respectively). These values were almost twice those seen on the other two leaf types (median oxidative activity was 0.03 μmol/h/cm² on both northern red oak and sycamore leaves, whereas median beta-glucosidase activity was 0.06 μmol/h/cm² for both). Median cellobiohydrolase activity was also similar for northern red oak and sycamore (0.01 μmol/h/cm²) but differed substantially between scarlet oak (0.015 μmol/h/cm²) and cherry (0.03 μmol/h/cm²). These differences are likely because of a wide range of physical and chemical characteristics that differ between decomposing leaf types [98]. As would be expected based on their roles in cellulose degradation [99, 100], activities of beta-glucosidase and cellobiohydrolase were correlated, but this correlation ranged from weak (R = 0.32) on scarlet oak to moderately strong (R = 0.79) on cherry.
Figure 4. Statistical distributions for microbial extracellular enzyme activity sampled across decomposing leaves. Five leaves of each species (A: cherry, B: scarlet oak, C: northern red oak, D: sycamore) were sampled for beta-glucosidase, cellobiohydrolase, and a combination of phenol oxidase and peroxidase activity. Solid lines for each leaf-enzyme combination indicate median values, with boxes indicating 25th and 75th quartiles, and whiskers representing 1.5x interquartile range. Most leaves showed high spatial variability in microbial activity even when outlying samples (open circles) were excluded.
Variability in microbial activity across the surface of each leaf type was determined using a linear model fitted to the data comparing each sample point to the mean. Results of this model yielded significance values of $p < 0.001$ for all three enzymes on each leaf type, confirming a significant degree of spatial variability. CV values ranged from 29.5 to 43.8 for the different leaf types, and on any particular leaf were similar for the three different enzymes. Several-fold variation in activity between sample points across the surface of individual leaves was common for all three enzymes, and the range of activities reported for a given leaf was substantial (Figure 4).

Because the median enzyme activity and the range of activities were similar between replicates, a single northern red oak leaf was chosen for a more detailed representation of the variability in activity of each of the three microbial enzymes across the leaf surface (Figure 5). All three enzyme measurements showed fine scale variability with individual sample points differing in activity even over distances of a few centimeters. This variability was typical of that seen in other leaves of northern red oak and in the other leaf types. Different spatial distributions of activity were visible for the three enzymes. Both beta-glucosidase and the combined oxidative enzymes showed distributions characterized by low background levels of activity with isolated “hot spots” of much higher enzyme activity. In contrast, cellobiohydrolase activity was intermediate on most of the discs sampled with intermittent points of both high and low activity. This finding is somewhat unusual given that activities of cellobiohydrolase and beta-glucosidase are more typically correlated [92], and the oxidative enzymes tend to show distributions different from those of the cellulases [92, 101]. However, most studies of microbial enzymatic activity sample at much larger scales than those examined here, and fine scale differences in environmental conditions across the leaf surface may be more important in influencing the distribution of some enzymes over others. Regardless, all three enzymes showed appreciable fine scale spatial heterogeneity in activity, even across the surface of a single leaf.

Figure 5. Spatial patterns of microbial extracellular enzyme activity across the surface of a northern red oak leaf. The images show the fine scale spatial distribution of beta-glucosidase, cellobiohydrolase and combined phenol oxidase/peroxidase activity measured across the surface of the same leaf. Sample points are represented by an open circle and enzyme activity is interpolated between them.
CONCLUSION

Aquatic microbial communities clearly function at fine scales and studies suggest that environmental heterogeneity at a level finer than that commonly examined in environmental microbiology can influence microbial community structure and function. This is apparent from planktonic communities, where differences in bacterial community composition and activity can occur between free-living cells and those associated with particles. Microorganisms in biofilms show clear spatial structure, largely because of environmental gradients that form within the biofilm matrix. Similar gradients occur with depth in sediments, although other fine scale environmental variation in sediment is also apparent. A survey of microbial activity and community structure in wetland sediment showed that particle size plays an important role in determining the microorganisms present, with substantially different bacterial communities being found on fine particles less than 250 μm compared to those greater than that diameter. These structural changes are also reflected in changes in microbial enzyme activity as particle size decreases. Microbial enzyme activity itself can be used to assess fine scale spatial patterns, such as those that occur on the surface of decomposing leaves. Activities of enzymes involved in lignocellulose degradation show high spatial variability over individual leaves, again demonstrating that microbial communities vary both structurally and functionally over finer scales than are usually considered in environmental microbiology. The impact of this variation on broader processes is unknown, but fine scale environmental variation should be considered in future surveys of microbial diversity and activity in aquatic environments.

REFERENCES


