BACTERIAL COMMUNITIES IN A NORTHEAST OHIO STREAM:
EFFECTS OF SUBSTRATE SIZE, ENVIRONMENTAL FEATURES AND TEMPORAL CHANGES

A dissertation submitted to Kent State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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May 2005
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Somebody said biology is messy; so is graduate school. This has been one of life's greatest challenges. It has been a sharp tool in the hands of Almighty God.

I owe a huge debt of gratitude and loyalty to my advisor, Dr. Laura Leff, who stood by me for 6 years. To my committee, Dr. Randy Hoeh, Dr. Mark Kershner, and Dr. Todd Royer, thank you for your suggestions and help with my research needs.

The most learning and joy came about because of lab partners and classmates. Real science occurred with them, at the bench, the computer, the hallway, or rarely (for me at least) even Ray's. How can we really ever explain to anyone else what it means to work side by side in frigid water, trying to hammer in or pull out some experiment? How can you tell someone, unless they have done it themselves that the all important key journal article your lab partner just copied for you is worth its weight in gold? Road trips, to places like Orlando, D.C., and Vancouver (for the 2 of us smart enough to go there in 2004, ask Melissa), just sweeten the pot! Here's to Hawaiian shirts and Birkenstocks! To my lab and classmates at KSU, you will always deserve the best I have to give.
DEDICATION

This dissertation is dedicated to my youngest daughter Leah Nicole. She feels she has earned part of this degree. I agree. Leah, this is a small thank you for who you are, and all you endured. I love you.

My son Courtney, daughter Dana and son-in-law Joe also provided support, humor and love to both Leah and me, thank you and I love you. Jesus Christ is the one who ultimately makes it all possible. He is and always will be the best I have to give.

Apparently this gets referred to as a “terminal” degree; an odd, but perhaps appropriate phrase for the blood, sweat, and tears of the last 6 years. Viable but not culturable is still discussed sometimes in our microbial ecology group but Chornobyl has yet to get off the back burner. More trips have been made to Ukraine and my passport now has a multiple entry visa; perhaps there will be time to pursue those water quality issues.

The epiphany on the banks of the Mississippi in St. Cloud, MN, still applies: “Impact comes from many directions and every ecologist who embraces the big picture knows all aspects are important in assessing the quality of the ecosystem. It is true in life. I am learning from the river to monitor input, drop loads too heavy to carry, and flow over or around the obstacles in the channel.”
CHAPTER I

General Introduction

The ubiquitous nature of biofilms in streams is well documented (Costerton et al. 1995) and they play an important role in nutrient and organic matter cycling in streams (Allan 1995). A review of the literature illustrates that the work done on bacteria in streams has largely focused on topics such as production (Marxsen 2001), metabolism (Romani and Sabater 2001), biofilm structure and function (Costerton et al. 1995, Lock 1993). These are important topics, but community composition is also relevant, and until the advent of current molecular techniques, has remained an understudied element in streams and rivers. An examination of species composition in stream sediment biofilms can help in our understanding of the processes driving their presence as well as those effects that emerge from their activities.

There are several potential biotic and abiotic influences on bacterial communities in streams (Pusch et al. 1998, Wetzel 1983). Several of these influences (biotic and abiotic) are discussed below and my research focused on what I believe to be the most significant influence: substrate (or streambed sediments) composition. In conjunction with substrate composition, including size and parent material, there are also other important associated abiotic effects (e.g., velocity and discharge). The biotic and abiotic influences described below may ultimately all be affected by substrate composition and associated factors.
Viruses

Little is known about the effect of viruses on lotic bacteria, particularly those associated with benthic biofilms. Lemke et al. (1997) studied the abundance and distribution of viruses in a Northeast Ohio lotic ecosystem (water, sediments, soil, leaves). The viral and bacterial numbers reported in this study were similar to those from marine studies. Of particularly relevance are the comparisons of main channel and pool counts, where the bacterial and viral counts were similar, suggesting that the impact of viruses is potentially similar across space. However, abundance may not be as important to understanding the role of viruses as examining viral and bacterial production rates.

Most work on the influence of viruses on bacteria has been done in marine ecosystems. Furthermore, the focus has been on the microbial loop (Fuhrman 2000) and processes affecting carbon and nutrient cycling (Bratbak et al. 1994). Figures vary but various studies report viral lysis of bacterial cells ranges from 2-20% per hour (Heldal and Bratbak 1991). Heldal and Bratbak also reported that 2-20% of bacteria are infected with mature virus particles while Suttle (1994) states 20% of bacteria are infected. Maranger and Bird (1995) compared coastal and freshwater lakes and observed a stronger relationship between chlorophyll $a$ and viral abundance than between chlorophyll $a$ and bacterial abundance. Yet, how does this relate to lotic systems? There are very few direct studies on the role of viruses in freshwater systems, especially streams and rivers.

The aforementioned studies have focused on the microbial loop and the effects on nutrient and carbon cycling, the question still remains: how are biofilm communities affected by viral lysis and is it an important process.
Protozoa

More work has been done on the fate of bacteria under protozoan grazing than on viruses, but research has focused on planktonic ecosystems (Pedros-Alió et al. 2000). Studies of sediments yield mixed results, with some researchers reporting significant effects from grazers and others dismissing their contribution as only a small factor (Hamels et al. 2001). The production of the exopolysaccharide matrix (EPS) in biofilms has a documented inhibitory effect on grazing (Lock 1993) and may cause a major difference in the effect of protozoan grazing on bacteria between sediment and planktonic habitats.

Abiotic influences on bacterial production (e.g., temperature, velocity) affect grazing rates as well as competition among grazers and these factors need to be considered when evaluating in conjunction with biofilm abundance and diversity. One study cites temperature as the single most important factor in these interactions (Vaque’ et al. 1994). They point out in their review of methods for studying grazing rates that temperature not only affects bacterial production but also chlorophyll concentrations and predator/prey sizes. Although temperature effects varied with methodologies, it was more of an influence for temperatures up to 18°C regardless of methods used. Since temperatures in Northeast Ohio streams, like the West Branch of the Mahoning, fall within this temperature profile, it is a variable to carefully consider in relation to protozoan grazing on bacteria.

Phenotypic traits like cell surface characteristics of bacteria may also be an important factor in protozoan grazing. One study by Matz et al. (2002) looked at the
influence of phenotype on feeding habits of nanoflagellates. They concur with other research that bacterial cell size is a strong influence but they also point out that cell surface characteristics and motility may be important.

One other abiotic factor important to consider when evaluating protozoan grazing on bacteria is sediment grain size. This is particularly relevant for the purposes of this study. The results of Hamels et al. (2001) show a breakdown of flagellate grazing pressure in fine sediments. Certainly, ciliates and amoeba may consume bacteria, but they suggest that viruses in these fine sediments may have more of an impact than protozoan grazers.

Perhaps the most important potential impact by protozoa may be related to changes in diversity rather than changes in abundance but there is little research at present to evaluate this possibility (Shikano et al. 1990). Abiotic factors (temperature and sediment size) influence protozoan and bacterial interactions, the question is how much and does it significantly alter the taxonomic composition of sediment bacterial communities?

**Invertebrates**

The number of studies on the effect of macroinvertebrates on benthic bacteria is limited. Most studies have focused on invertebrate use of bacteria as a food source.

One experiment observed reduced bacterial abundance from biofilms associated with increased numbers of blackfly larvae attached to experimental clay pots (with
nutrient additions) in a small trout stream (Santmire 2000). Blackflies were responsible for 91% of bacterial ingestion in another small stream as reported in Hall et al. (1996).

Other filter feeders that may consume bacteria include bivalves such as Corbicula (Silverman et al. 1995), as indicated in various laboratory observations. Corbicula are filter feeders but can also potentially consume benthic bacteria via pedal feeding and alter bacterial distribution (Leff and Leff 2000). However, according to McEwen and Leff (2001) bacteria ingested by Corbicula do not readily colonize the gut. Filter feeders (e.g., blackfly larvae and mussels) are more apt to ingest planktonic bacteria than benthic bacteria. Attached blackfly larvae, such as those observed on the clay pots (Santmire 2000), may have been more of a disturbance factor, due to the attachment.

Other studies show mixed results regarding ingestion of benthic bacteria by macroinvertebrates and what effect they have on populations of benthic bacteria. Austin and Baker (1988) report Ephemera digested significant amounts of two species of bacteria (Acinetobacter and Flavobacterium/Flexibacter), while an earlier study (Lawson et al. 1984) postulated that an obligate detritivore, Tipuladae utilized only a small percentage of the bacteria.

Algae

The interactions of planktonic bacteria and algae (marine and freshwater) have been well studied for more than twenty years (e.g., Cole 1982, White et al. 1991), while the interactions of benthic bacteria and algae have been less well studied (Sobczak 1996). The strength of the connection between bacteria and algae has also been debated (Haack
and McFeters 1982, Stock and Ward 1989, Findlay et al. 1993). The work of Winterbourn (1990) shows the importance of biofilms to primary and secondary production. It is often presumed that algal exudates provide important dissolved organic matter (DOM) to bacteria, but this could depend on the maturity of the biofilm (Cole 1982). Kaplan and Bott (1989) noted changes in available DOM related to diel algal changes while Findlay et al. (1993) believe the bacteria could rely totally on allochthonous DOM. This variation in bacterial response could likely be due to the maturity and composition of the biofilm (Blenkinsopp et al. 1991), with some biofilms relying more on algal DOM compared to those found in more oligotrophic streams or in locations where shear stress, discharge and shading would be more of a factor.

With regard to competition between algae and bacteria at the sediment-water interface, bacterial communities may out compete algae since they are able to use a variety of sources of organic matter and nutrients (Wetzel 1983, 2000). Given the potential for bacteria to also use algal exudates this minimizes the negative effect algae may have on bacteria.

**Nutrients**

There have been studies for at least 20 years or more that look at the effects of nutrients in streams (e.g., Meyer 1979), including research that addresses biofilm response to nutrients (Haack and McFeters 1982). However, its influence on changes in composition of sediment bacterial communities is not well understood.
As with potential influences of protozoa and viruses, the literature once again does not seem consistent regarding the influence of nutrients on microorganisms. For example, a study by Marshall (1988) observed detachment of bacteria under oligotrophic conditions while James et al. (1995) found the opposite to be true. An in situ experiment documented lower abundance with combination nutrient additions (nitrate and phosphate) compared to single nutrient additions and the control (Santmire 2000).

Results from a study by Mohamed et al. (1998) investigating biofilms in a Canadian River suggest phosphorus limitation is important to lotic bacteria. However, their methodology used planktonic bacteria as inocula for cultures to develop biofilms and then these cultures were amended with nutrients. This does not necessarily reflect the true biofilm bacterial community in situ. Nevertheless, their study showed a correlation between combinations of nutrients and increased protist grazing thus indicating the possible link between bacterial production and increased grazing pressure as it relates to nutrient additions (particularly in combination).

In another study evaluating nutrients and microorganisms in stream benthos, Bonin et al. (2000) also observed that phosphorus might be a limiting factor. They saw seasonal correlations between microbial activity and total phosphorus. Interestingly, there was more phosphorus in the stream at high elevations but less microbial activity, which was the opposite of what they predicted. They suggested that either substrate quality or temperature might be the limiting factors.
**Organic Matter**

Organic matter enters streams as both allochthonous (e.g., plant material) and autochthonous (e.g., algal material) input (Allan 1995). It plays a dual role, providing a carbon source and also providing, in some cases, an organic substrate for bacteria. Canopy coverage affects algal biomass and litter input that in turn has been shown to affect bacterial production (Rier & Stevenson 2001).

Dissolved organic matter (DOM) is the dominant organic matter pool in streams and moves at a rate concomitant with water molecules (Allan 1995). On the other hand, particulate organic matter (POM) is more closely linked with discharge and storm events, being both stored and exported (Cummins et al. 1983).

Different fractions of the organic matter pool vary in availability to bacteria (Freeman et al. 1990). These differences could effect the bacterial community composition yet these are understudied areas (Gao, personal communication). The link between organic matter and the physical regime of the stream (discharge and velocity) makes the influence of organic matter and its relationship to substrate and bacterial diversity an important consideration. Studies have shown that the available organic matter from surface water supports at least 50% of the benthic bacterial production (e.g., Bott et al. 1984).

**Velocity, Discharge, Groundwater Interface**

The rate at which water flows and the amount of water flowing through any given stream define a major constituent of the baseline parameters of a lotic ecosystem. Ward
and Stanford (1983) applied Connell’s Intermediate Disturbance Hypothesis (1978) to streams citing evidence that anthropogenic disturbance may cycle a stream towards equilibrium rather than maintaining the diversity naturally occurring in streams with seasonal variations. Their statement that undisturbed streams are in fact disturbed is worth serious consideration.

The seminal work of Vannote et al. (1980), referred to by virtually all stream ecologists at one time or another, also proposes a change in diversity along the River Continuum and this is intimately associated with both the rate at which the water flows along a river and the amount of water passing through the system. These two key components, velocity and discharge, help determine the size of the sediments along any given stream reach. Other factors that influence the sediment distribution are overall channel geomorphology.

The research of these scientists recognizes the importance of physical features to ecology, but at their inception, molecular techniques were not what they are today and microorganisms were treated as a black box in streams. This is a critical area of study for microbial ecologists who can draw on both the historical understanding available and the ability now to evaluate the influence of physical features on composition of bacterial communities.

**Temperature, pH and Dissolved Oxygen**

The fact that temperature affects microbial activity is not disputed (Kaplan and Bott 1989, Marxsen 2001), but there is not always consensus as to how much it affects
the activity and changes of bacterial communities in streams. Claret et al. (1998) associated temperature with nutrient fluxes (e.g., denitrification in the sediments) and reported more activity in biofilms during the warm summer season.

Tank and Winterbourn (1995) found little evidence to support reduced microbial activity on organic substrata under lowered pH conditions, although this does not specifically address the question of changes in the structure of the microbial community itself.

Smaller streams without significant impact from pollutants (e.g., sewage effluent, agricultural run off, siltation) generally have close to saturation levels of dissolved oxygen (Allan 1995). Diel fluctuations, with reduced levels of oxygenation can occur in larger rivers when there is more biological demand, or more human impact (Wetzel, 1983, 2000). Sediment biofilms will not be diminished by changes in oxygen levels, but the metabolic activity or species dominant in the sediments can change.

Substrate Features

Both the parent material and the size of the substrate that form the benthic sediments play a crucial role in the biology of a stream. Size and shape of sediment particles is determined by parent material and physical and chemical action of the environment over time (Marxsen 2001). The ability of the particles to pack and the ability of a stream to do work (i.e., move sediments downstream) is an underlying factor determining changes in bacterial communities in sediments. Claret and Fontvielle (1997)
recognize seasonal variations in microbial activity but believe hydrodynamic factors can modify biofilms and how they use nutrients.

Recent work by Romani and Sabater (2001) maintain that there was a community difference in bacterial activity and biomass between epilithic biofilms and episammic biofilms. The epilithic biofilms had increased biovolume and biomass and more seasonal stability, while the episammic biofilms had more metabolic activity and abundance. The question remains, are these differences in metabolic activity related to changes in the bacterial community composition associated with the varying size of the sediments?

**Summary of Potential Influences**

There are multiple potential influences on bacteria in stream sediments but there is often disagreement as to the relative impact of a particular biotic or abiotic factor. There are often multiple effects (e.g., temperature affecting protozoan grazing; substrate size affecting types of communities) and these effects may vary across lotic ecosystems. In fact, they may vary within the same ecosystem, or stream reach, at different times or seasons. Sediments are key to cycling of nutrients and organic matter, bacteria play an important role in these processes, and there is much work yet to be done.

Of the biotic factors possibly influencing bacteria (i.e. viruses, protozoa and invertebrates), the literature is not convincing as to their role in sediment biofilms. That these organisms affect bacteria in steams is not in dispute, how much influence, still requires more investigation.
There are also often conflicting reports as to the effects of nutrients on microbial communities. With organic matter, there is no question it plays an important role. However, I am not convinced that either is a driving force in changes in sediment biofilm communities. Flowing water and underlying geomorphology define any stream parameter, and I believe these factors set the context for the bacterial communities that dwell therein. It is with this in mind I proposed the following research. There is a need to establish bacterial communities in streams, i.e., what species are actually present in the stream, particularly in the sediments, since this is the location of most activity for microbial cycling of organic matter and nutrients.

With current molecular techniques, such as oligonucleotide probes targeting ribosomal RNA at taxon-specific and species-specific levels, it is now possible to evaluate bacterial communities without culturing them (Amann et al. 1990). Coupled with the importance of the sediments in streams, herein referred to either as sediment, particles or substrate, all referring to the inorganic particles in the stream, this research focused on the importance of particle grain size and its effects on the species colonizing the sediments in a reach of the West Branch of the Mahoning River in Northeast, Ohio. Other features, i.e., temporal changes, organic matter addition and effects of velocity, with regard to particle size, were also evaluated. Understanding the influence of the streambed sediments on colonizing sediment bacteria has potential to understand how manipulation of streams might affect in situ populations.
Overview of Research Objectives

The research represented by this dissertation spans 4 years (2000 through 2004), investigating bacterial assemblages and the potential influences of selected environmental features on those populations. The purpose of the study in Chapter II represents a yearlong study of three important microhabitats in a reach of the West Branch of the Mahoning River. This allowed me to examine the extent of seasonal changes in different habitats and seek relationships among environmental variables and bacterial abundance.

Chapters III-V focus on sediment grain size and its influence on bacterial biofilm communities in the sediments. Chapter II describes a seasonal survey of sediments and cobbles over one year (late summer 2003 through mid-spring 2004). In this chapter, bacterial communities on sediments of different sizes were compared.

The research presented in Chapter IV was designed to empirically examine the effects of substrate size on bacterial communities in sediments. Additionally, two other potential influences were examined: organic matter addition and spatial differences, in this instance, bacterial assemblages on artificial substrate incubated in a riffle versus and adjacent pool were compared. The artificial substrate used for these experiments were three sizes of glass beads and unglazed tiles that permitted quantification of bacterial numbers on both per gram and per surface area basis.

The research presented in Chapter V was designed to investigate the possibility that bacterial communities on one substrate size are somehow specialized in their ability to grow on that particular size class. To accomplish this, two of the glass bead sizes (0.1 mm and 5.0 mm) were used that were shown to have significant differences in bacterial
colonization from the experiments presented in Chapter III. Packets of beads were incubated for one month in the stream, bacteria removed and then used to seed microcosms containing with the other size substrate and allowed to incubate for 6 days.

Throughout this research, total bacterial abundance was accomplished via epifluorescent microscopy using 4,6-diamidino-2-phenylindole (DAPI) stain (Porter and Feig 1980). In order to evaluate bacterial assemblages at multiple taxonomic levels, fluorescent in situ hybridization (FISH) targets cellular ribosomal RNA (rRNA) hybridized with a taxon-specific or species-specific fluorescently labeled probe (Amann et al. 1990). Where appropriate, physical and chemical data were collected to evaluate their possible correlation with bacterial communities in the stream.
References


Bott, T. L. and M. A. Borchardt. 1999. Grazing of protozoa, bacteria and diatoms by


CHAPTER II

Temporal changes in the bacterial assemblage of a Northeast Ohio stream: a comparison of community and population-level responses

Abstract

A limited number of studies have documented temporal changes in multiple habitats in streams. In this yearlong study of the West Branch of the Mahoning River in Northeast Ohio, USA, bacterial assemblages in water, leaves, and sediments were examined. Bacteria were enumerated using 4,6-diamidino-2-phenylindole (DAPI) and fluorescent in situ hybridization (FISH) using taxon-specific probes for the Domain Bacteria and Burkholderia cepacia. Physical and chemical variables were also monitored. Total bacterial abundance in water (based on DAPI staining) peaked during October 2000 and July 2001; while on leaves, total abundance peaked in January then declined through April with a second June peak. The peak in sediments was during October 2000 and numbers did not differ significantly between the pool and riffle. Domain Bacteria numbers also exhibited significant temporal changes but the seasonal patterns tended to differ from those based on DAPI staining. Abundance of B. cepacia exhibited significant temporal changes on leaves but not in water and sediments.

Submitted to Hydrobiologia by Judith A. Santmire and Laura G. Leff
Contrary to other studies, no significant correlations were seen between the bacteriological and the physical/chemical variables measured. Spring run off seems to have been a factor in temporarily reduced counts on leaves and sediments. Conversely, on this date, bacterioplankton numbers were higher, perhaps due to allochthonous inputs of organic matter (e.g., leaves). Based on prior studies, we expected the pattern of temporal change in bacterial numbers to vary among habitats. However, there was a surprising lack of differences between pool and riffle sediment and no significant correlations between bacteriological and abiotic variables, other than a possible response scouring with increased discharge at the end of winter. This likely reflects the ability of bacteria to persist under low temperature/nutrient conditions and varying flow regimes. The ability of *B. cepacia* to maintain fairly constant populations, in contrast to the overall assemblage, likely reflects the extreme versatility of this organism.
Introduction

Integration of multiple dimensions in lotic ecosystems (e.g., temporal, lateral, vertical) is necessary to provide a well-constructed framework for understanding streams (Ward, 1989). In keeping with this need to examine patterns across dimensions, studies of bacterial abundance in streams have documented spatial and temporal variations in specific habitats, such as water, sediments, and leaves (e.g., Leff et al., 1999; Lemke & Leff, 1999, Maamri et al. 1999).

Factors that influence bacterial abundance vary among habitats. For example, abundance in water may be related to allochthonous input of cells (Sponseller & Benfield 2001) or temperature changes (Freeman et al. 1990). In contrast, abundance on leaves can vary in relation to residence time, decomposition rates, and grazing (Tank & Webster 1998, Golladay & Sinsabaugh, 1991). In sediments, scouring, benthic organic matter, and grazing, may be important factors for bacterial communities (Hillebrand et al. 2002, Wainright et al. 1992).

Bacterial abundance in streams is typically reported based on total bacterial counts, using fluorescent stains such as 4,6-diamidino-2-phenylindole (DAPI; e.g., Ping & Leff 2004, Liu & Leff 2002, Halda et al. 2001) or acridine orange (e.g., Mathias et al. 1995, Maamri et al. 1999). Based on these approaches, temporal changes in total numbers have been reported and it has been demonstrated that seasonal changes are more pronounced in the water column than in sediments (Leff et al. 1999, Leff et al. 1998, Lamberti & Resh 1987).
In some studies of seasonal changes in bacterial numbers, specific bacterial taxa have been enumerated (e.g., Liu and Leff 2002). This can be accomplished via fluorescent in situ hybridization (FISH) in which fluorescently labeled taxon-specific probes are hybridized with rRNA in intact cells (Amann et al., 1990). The temporal patterns that are depicted based on enumeration of total bacteria versus specific taxa can be very different (e.g., Liu & Leff 2002). Compared to total bacterial counts (e.g. using DAPI) different patterns may arise depending on the specific taxon targeted.

Leff et al. (1999) looked at spatio-temporal variation in abundance (based on cultivation) of a specific species (i.e., *Burkholderia cepacia*), noting that at the species level differences were observed that were not reflected in total bacterial abundance. *B. cepacia* is common in streams and has been relatively frequently studied in lotic systems (McEwen et al. 2001, Janakiraman & Leff 1999, Lemke et al. 1997, Leff et al. 1995). Besides occurring naturally in streams, *B. cepacia* is an opportunistic plant (Kawamoto & Lorbeer 1974) and animal, often human, pathogen (Hutchison et al. 1998, Falkow et al. 1992), and readily attaches to surfaces, particularly mature biofilms (Al-Bakri et al. 2004). *B. cepacia* is also known for its antibiotic resistance, making this microorganism both environmentally and medically of interest, especially in biofilms (Stewart and Costerton 2001).

The purpose of this study was to examine temporal changes in bacterial abundance in three stream habitats: water column, conditioned leaves, and sediments (pool and riffle). Water in streams is transient, compared to leaves that fall into the stream and benthic sediments. For example, as leaves decompose, they release organic
matter and nutrients, while providing substrata for associated biofilms and sediments. Community-level, and population-level responses were compared by enumerating total numbers of bacteria (using DAPI), and members of the Domain *Bacteria* and *B. cepacia* (based on FISH).

**Methods**

*Study Site*

The West Branch of the Mahoning River is a 4th order stream within the Mahoning River watershed in Northeast Ohio (Latitude 41°09'41", Longitude 81°11'50"OH EPA Technical Report MAS/1995-12-14). Samples were collected from a reach 71 meters in length comprised of a pool, riffle and run, with an average width of 16 meters. Riparian vegetation consists of hardwoods, predominately, sycamore, maple and oak, with approximately 90% canopy coverage during summer months.

*Sample Collection and Preservation*

Samples (in-stream senescent leaves, water, and sediment) were collected from October 2000 to October 2001. Weekly sampling was performed from October 2000 through November 2000 and then varied from biweekly to monthly depending on the season and stream conditions, on a total of twenty-one dates. Triplicate samples of in-stream conditioned leaves (N=3), water (N=3) and sediments (N=3 each from the riffle and pool) were collected, returned to the lab and preserved with phosphate buffered, 8%
paraformaldehyde. Sodium pyrophosphate was added to leaves and sediment samples that were then sonicated for 5 minutes (Branson 2210 Sonicator, Danbury, CT) and stored at 4°C.

Supporting physical and chemical data were also measured on each date. Temperature, pH, conductivity and turbidity were determined in the field and water samples (N=3) were collected and kept on ice until returned to the lab to measure nitrate-nitrogen and soluble reactive phosphorus (SRP). Temperature and pH were measured with an Oakton pH/mV/°C Meter. Specific conductance was measured with a Hach Conductivity/TDS Meter (Model 44600). Turbidity was measured with a Hach 2100P Turbidimeter (46500-00) while nitrate/nitrite-nitrogen and SRP were measured with Hach kits, DR100 Colorimeter Nitrate (Low Range) (41100-13) and Hach DR 100 Colorimeter Phosphorous, Reactive (41100-16), respectively.

A portion of each sediment and leaf sample was used to determine dry weight and ash-free dry mass (AFDM). Dry weight was determined by drying over night at 104°C (Baxter TempCon Oven, Scientific Products). AFDM was determined by placing dried samples in a muffle furnace (Thermolyne 30400 Furnace) for 5 hours at 550°C. Particle size fractions of sediments were determined through manual sieving using U. S. Standard Sieves.

Bacterial Abundance

Total numbers of bacteria were determined via epifluorescent microscopy using DAPI stain (Porter and Feig 1980). Samples were concentrated on black 0.2 µm
polycarbonate filters (Poretics, Livermore, CA), stained with DAPI (3-4 minutes), and rinsed using sterile, filtered water.

In FISH, cellular rRNA is hybridized with taxon-specific fluorescently labeled probes (Saylor and Layton 1990). The probes used for this study targeted the Domain *Bacteria* (EUB 338, 5’-3’ GCTGCCTCCCGTAGGAGT; Amman 1990) and *B. cepacia* (5’-3’ CCTCTGTTCCGACCA; Braun-Howland et al. 1993). Briefly, FISH was accomplished as in Lemke et al. (1997) by concentrating samples onto 0.2 µm Anodisc filters (Whatman, Maidstone, UK) that were rinsed with autoclaved, distilled, deionized water (ddH2O) and 0.1% Nonidet P-40 (Sigma). Filters were placed in Petri dishes and 40 µl of Texas red labeled probe (Sigma Genosys) at 5 ng/µl final concentration in hybridization buffer (6x SSC, 0.02 M TRIZMA base with pH 7, 0.1% SDS and 0.01% polyadenylic acid) was applied to each Anodisc then incubated for 4 hours at appropriate temperatures (48°C for Domain *Bacteria* and 49°C for *B. cepacia*).

Once incubation was complete, filters were rinsed with 800 µl of wash buffer (.9 M NaCl, 0.02 Tris (pH7.2), 0.1% SDS), and returned to fresh Petri dishes where 80 µl of wash buffer was applied to each filter. This process was repeated then filters were rinsed with 800 µl of ddH2O and placed on slides for enumeration via epifluorescent microscopy.

**Statistical Analyses**

Analysis of variance (ANOVA) was performed using SAS (SAS Institute Inc., Cary, NC, 1999). Two-way ANOVAs were performed on the pool and riffle sediments to
check for significant differences among sediments in the two regions and determine if
results could be combined. Based on these analyses, pool and riffle sediments did not
significantly differ in bacterial abundance and thus the values from these two regions
were combined. Subsequently, analyses reported are one-way ANOVAs that compare
sampling dates for each bacteriological variable measured. Correlation analyses were
performed between abiotic environmental variables and bacterial abundance.

Results

Physical and Chemical Data

Temperature varied over the year from a low of 0.75 °C on 9 December 2000, to a
high of 18.96 °C on 9 July 2001. The pH varied from a low of 7.20 on 20 January 2001
to a high of 9.11 on 27 April 2001. Conductivity ranged from 230 µS/cm (9 July 2001)
to 760 µS/cm (9 December 2000) and turbidity varied from 1.55 NTU (28 October 2000)
to 19.38 NTU (17 March 2001). The 17 March 2001 sampling date corresponds to the
time of spring run-off and is reflected in the environmental variables measured. Overall,
there was little variation in SRP with a low of 0.12 SRP mg P/L on two dates in
November 2000 (18th and 25th) and a high on 9 December 2000 (0.37 mg P/L). Most
nitrate/nitrite-nitrogen readings were less than 0.1 mg N/L and the highest measurement
of 0.35 mg N/L was recorded 20 January 2001, with a second high concentration of 0.32
mg N/L on 17 March 2001.
The percent organic matter (%OM), determined using AFDM, varied more with the leaves, 16.05% (11 November 2000) to 32.47% (13 April 2001), than the sediments, which ranged from negligible (<0.00%) on 27 April 2001 to 8.46% on 17 March 2001 (Table 1). Sediment particle sizes were predominantly (> 70%) greater than 1.0 mm in diameter for both the riffle and the pool areas with approximately 20% in the 0.5 mm range category and < 10% in the 0.1 mm range category (data not shown).

**Bacterial Abundance**

Total numbers of bacteria (based on DAPI staining) were significantly different among dates in all habitats (p < 0.0001 for water and sediments; p = 0.03 for leaves) and peak abundance occurred at different times for each habitat (Figure 1). There were two peaks for water, one on 28 October 2000 and a second on 9 July 2001 (Figure 1A). The peak abundance for the leaves was on 20 January 2001 (Figure 1B).

In sediments, there were no significant differences in abundance, regardless of enumeration method between the riffle and the pool (based on a two-way ANOVA, p = 0.34 for DAPI, p = 0.78 for Domain Bacteria, p = 0.66 for B. cepacia) and thus the data were combined. Overall, in sediment, the peak in total bacterial abundance was on 28 October 2000, not peaking again the remainder of the year (Figure 1C). Abundance on sediments was less variable than in the other two habitats.

The abundance based on FISH with the Domain Bacteria probe presents a slightly different picture than numbers based on DAPI staining, but there were also significant differences temporally for all three habitats (p < 0.0009 for water, p < 0.0001 for leaves
and sediments, Figure 2). The sole peak in abundance for water was on 4 November 2000 (Figure 2A). For leaves, the peak was on 9 December 2000 with a second smaller peak on 25 September 2001 (Figure 2B). The sediments showed more variation over the year with the highest abundance on 9 December 2000 as with the leaves, and a smaller peak on 4 November 2000 (Figure 2C).

Using the species-specific probe for *B. cepacia*, another temporal pattern emerges: for the water, there were fairly stable numbers over the year and no significant differences among dates were observed (p = 0.76, Figure 3A). For the leaves, there was significant changes over the year (p = 0.04, Figure 3B), as was seen with DAPI and Domain *Bacteria* counts, but the greatest numbers were on 4 November 2000 and 18 June 2001. For the sediment, peak *B. cepacia* abundance was on 28 October 2000, which was also noted for DAPI, but there were no significant temporal differences (p = 0.58, Figure 3C). On several dates, the variability among replicate samples was higher than observed for the other enumeration methods.

When correlation analyses between bacterial abundance and environmental variables were performed, no significant relationships were detected for any of the bacteriological variables (DAPI, Domain *Bacteria* or *B. cepacia*) and the environmental variables measured. Flooding seems to have been a factor in temporarily reduced counts on leaves and sediments, which was reflected in DAPI counts for sediments and FISH counts for both leaves and sediments. Conversely, during flooding there were greater numbers of *B. cepacia* in the water.
Discussion

To fully examine temporal variation in bacterial communities, assessments targeting different habitats and taxonomic levels are needed. In this study, responses at the community level (based on DAPI staining and FISH with a Domain Bacteria probe) differed from population level responses (based on FISH with the B. cepacia probe). These differences in turn depended on the habitat from which samples were drawn.

Relative to the other two habitats, the water column is more transient, and the variation in numbers in water is likely to be strongly influenced by differences in allochthonous inputs among dates (Leff et al. 1998, Liu & Leff 2002).

For bacteria in the water column, total abundance followed a seasonal pattern similar to that observed at some sites sampled in the Cuyahoga River in another study (NE Ohio; Leff et al. 1999) with highest numbers in June and lower numbers at the time of spring runoff in March. Likewise, a similar trend was found in two Northeast Ohio streams (including the same one sampled in this study; Leff et al. 1998). In another study on the Cuyahoga River that contrasts with this study and that of Leff et al. (1999), higher total numbers were observed during spring run-off (March) compared to the other dates examined; this trend was attributed to influxes of allochthonous cells (Liu & Leff 2002).

Although a number of studies have looked at temporal changes in the water column, fewer studies have examined such changes in sediments and on leaves. For leaves, there was a lag period before peak abundances of bacteria (based on DAPI and FISH with the Domain Bacteria probe). The peak abundance on leaves occurred when temperatures were comparatively low and this lag is likely accounted for by the time
needed for a fully developed fungal community, an important factor that can enhance bacterial abundance (Sponseller & Benfield 2001). While both total and Domain *Bacteria* counts followed the same general trends over time, the variances in total counts (based on DAPI staining) tended to be greater. This might be due to colonization differences at the microhabitat scale since leaf samples were of mixed species (predominantly maple and sycamore) and the amount of material of a given species changes over time in relation to their decay rate (Ostrofsky 1997).

Numbers of DAPI stained cells and those detected by FISH with the Domain *Bacteria* probe generally followed different temporal patterns. As in other studies, total bacterial counts were higher than with FISH counts using the Domain Bacteria probe (e.g., Liu & Leff 2002). FISH targets rRNA, and thus may more accurately reflect abundance of active cells at the time of sampling rather than total cells present (Poulsen et al. 1993).

In this study, the % difference between Domain *Bacteria* and DAPI counts averaged 49% for water, 10% for sediment, and 32% for leaves. The large differences observed among the three habitats are consistent with the survey of Bouvier and del Giorgio (2003), in which values are reported for 19 different freshwater systems. They report that % difference between Domain *Bacteria* and DAPI in sediments tends to be less than 40% (range from 1-100%). Overall they report that on average 56% of cells present in a freshwater sample are detected with EUB338. In this study, overall, the percentage averaged among the 3 habitats was 30%, lower than reported in Bouvier and del Giorgio (2003), but within the range.
*B. cepacia* was selected for examination of a population-level response because prior studies suggest that it is likely to be well represented, or at least present, in all three habitats (water, leaves and sediments) over the course of a year (Lemke et al. 1997, Lemke & Leff 1998, Janakiraman & Leff 1999). What was most noticeable about this species was that few seasonal changes were observed in the water column and the sediments. The ability of *B. cepacia* to use a varying types of organic matter may account for the lack of temporal changes in numbers (Palleroni 1975). In this study, there was a similar rise in numbers in the water column during spring runoff, as in a study on the Cuyahoga River (Leff et al. 1999), which found elevated levels of *B. cepacia* at some sites in spring; however, in the present study, differences among dates were not significant. The lack of seasonal changes in the water column differs greatly from a study in the same stream where culturable *B. cepacia* were enumerated (Leff et al. 1998). In that study, culturable *B. cepacia* were not detected in water in summer and peaked in November. The current study demonstrates that *B. cepacia* is present throughout the year and thus differences documented in Leff et al. (1998) are likely due to seasonal changes in being culturable, not overall numbers.

In contrast to the water and sediments, there were significant temporal changes in *B. cepacia* numbers on leaves, and also a high amount of variability. *B. cepacia* exhibits the ability to use a variety of substrates and has very high genetic diversity with 9 currently known genomovars (Al-Bakri et al. 2004). Temporal changes in numbers of cells on the leaves might be due to changes in the type of leaf substrate present or small-scale differences in flow, resulting in a patchy distribution of this species.
Prior studies suggest that temporal changes, among bacterial communities of multiple habitats, are to be expected. What was more surprising was the lack of differences between pool and riffle sediment samples and the overall lack of correlation with any of the abiotic features measured, other than a noticeable response to spring run off. This likely reflects the ability of bacteria to grow under a variety of conditions and reinforces notions regarding the ability of stream microorganisms to survive under low nutrient conditions and varying flow regimes (Morita 1997).

The ability of *B. cepacia* to maintain fairly constant populations in various habitats, in spite of the large seasonal changes in stream features, is of potential importance given its role as a pathogen of both plants and humans. The extreme versatility of this organism and its genetic diversity may contribute to its success in lotic ecosystems.
References


In: Clarke P. H., & M. H. Richmond (eds) Genetics and biochemistry of

Ping, X. & L. G. Leff, 2004. Longitudinal changes in the benthic bacterial community of
the Mahoning River (Ohio, USA). Hydrobiologia 522: 329-335.

Porter, K. G. & Y. S. Feig, 1980. The use of DAPI for identifying and counting
aquatic microflora. Limnology and Oceanography 25: 943-948.

Poulsen, L. K., G. Ballard, & D. A. Stahl, 1993. Use of rRNA fluorescence in situ
hybridization for measuring the activity of single cells in young and established


Sponseller, R. A., & E. F. Benfield. 2001. Influences of land use on leaf breakdown in
Southern Appalachian headwater streams: a multiple-scale analysis. Journal of the


Table 1. Physical and chemical data for West Branch of the Mahoning River, October 2000 through October 2001. N=3 for 10/21/00 through 12/9/00; N=5 for 1/20/01 through 10/16/01, (±SE). ND = no data. BDL = below detection limits. For 11/25/00, N = 1 with no SE, for sediment AFDM.
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<th>Date</th>
<th>Nitrate/Nitrite (mg N/L)</th>
<th>SRP (mg P/L)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Conductivity (µS/cm)</th>
<th>Turbidity (NTU)</th>
<th>AFDM (% OM Leaves)</th>
<th>AFDM (% OM Sediments)</th>
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</thead>
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<td>10/21/00</td>
<td>BDL</td>
<td>0.27 (0.03)</td>
<td>7.10 (7.54)</td>
<td>12.23 (0.27)</td>
<td>520 (1)</td>
<td>2.11 (0.23)</td>
<td>22.26 (2.99)</td>
<td>ND</td>
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<td>BDL</td>
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<td>8.06 (9.24)</td>
<td>12.63 (0.03)</td>
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<td>25.00 (2.49)</td>
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Figure 1. Total bacterial abundance based on DAPI staining in 3 habitats: A. Water (N=3, ± SE) B. Leaves (N=3, ± SE) C. Sediment (N=6 ± SE). There were no samples for 10/21/00 for sediments. There were no senescent leaves for 7/19/01 and 8/15/01.
Figure 2. Abundance of cells hybridizing the Domain *Bacteria* probe in 3 habitats: A. Water (N=3, N=2 on 10/28/00 and 11/11/00 ± SE) B. Leaves (N=3 ± SE) C. Sediment (N=6 ± SE). There were no samples for 10/21/00 for sediments. There were no senescent leaves for 7/19/01 and 8/15/01.
Figure 3. Abundance of cells hybridizing the *B. cepacia* probe in 3 habitats: A. Water (N=3, ±SE) B. Leaves (N=3, ±SE, N=2 for 6/18/01, ±SE) C. Sediment (N=6, ±SE).

There were no samples for 10/21/00 for sediments. There were no senescent leaves for 7/19/01 and 8/15/01.
CHAPTER III

Differences in bacterial communities among stream sediments of different sizes

Abstract

Sediment features may play a major role in structuring benthic bacterial community structure. In this study, sediment samples were collected on four dates over the course of a year from a Northeast Ohio stream and fractionated into different size classes. Abundance of bacteria of different taxa in the samples was determined using fluorescent in situ hybridization. Generally, differences in abundance among the size classes were small in comparison to the large seasonal changes observed. These seasonal changes differed greatly among taxa with some exhibiting peak abundance in spring (Domain Bacteria, α-Proteobacteria) and others peaking in summer/fall (γ-Proteobacteria and the Cytophaga-Flavobacterium). At the species level, the abundance of Burkholderia cepacia and Acinetobacter calcoaceticus were highest in summer on sediments of all sizes. Whereas seasonal changes appeared to be more of a factor driving community variation, there were significant differences in abundance on sediments from...
the different categories. Differences in responses among the taxa examined likely resulted from innate variation in features, such as nutrient requirements.

**Introduction**

Benthic processes play a dominant role in stream bacterial ecology and the features of the sediments potentially greatly impact the microbial community (Edwards et al. 1990, Romani and Sabater 2001). Within the sediments, there is often a heterogeneous mixture of particles of different sizes, origins, and surface features. These differences potentially impact the number of bacteria as well as the composition of the bacterial community (Eisenmann et al. 1999).

Besides spatial features, temporal dynamics are important factors in lotic ecosystems (Ward 1989) and benthic bacterial community composition can change dramatically over the course of a year (Olapade and Leff 2004). For example, increases in discharge can have a rapid impact on stream biota through scouring and abrupt changes in streambed morphology (Holmes et al. 1998). Likewise, seasonal changes in temperature can affect grazing rates on bacterial populations (Vaque et al. 1994). Marxsen (2001) examined bacterial production in varying sediment types and demonstrated that temperature and discharge were the most important factors affecting bacterial production in a stream. Marxsen also observed lower production rates on the sandy grains compared to the coarse particles and that flooding had a greater impact on the more coarse substrate. Overall, Marxsen (2001) demonstrated that seasonal differences, as well as difference in sediment grain size, impacted bacterial production. Thus the premise of the present study is that seasonal changes as well as sediment grain
size will impact the abundance of bacteria of different taxa and thus alter the community structure.

In this study, the potential influences of substrate grain size were examined by comparing bacterial communities among substrate of different sizes collected on four dates over the course of a year (once each season). To accomplish this, sediment samples (collected from a stream in Northeast Ohio, USA) were fractionated into size classes and the abundance of bacteria of different taxa were enumerated using fluorescent in situ hybridization (FISH). In FISH, taxon-specific oligonucleotide probes that target rRNA of cells are used to differentiate among cells of different taxa (Amann et al. 1990).

Taxa examined were selected based on their use in prior studies, are abundant in lotic systems, and exhibit high degrees of spatio-temporal variation in abundance (Olapade et al. in press, Olapade and Leff 2004, Xu and Leff 2004, Liu and Leff 2003). Specifically, members of the Domain Bacteria, the α-Proteobacteria and γ-Proteobacteria and the Cytophaga-Flavobacterium cluster were enumerated. Species-specific probes used were for Burkholderia cepacia and Acinetobacter calcoaceticus. Prior studies suggest that these taxa potentially differ in their responses to environmental conditions (e.g., Olapade and Leff 2004) and thus it is hypothesized that different taxa will exhibit different seasonal patterns and show various responses to sediment grain size.
Methods

Study Site

Samples were collected from the West Branch of the Mahoning River, a 4th order stream in Northeast Ohio (Latitude 41°09'41", Longitude 81°11'50"OH EPA Technical Report MAS/1995-12-14), from the riffle of a 30 m long by 14 m wide reach with an average depth of 20 cm over the year. This site has been the subject of several studies on bacterial ecology (e.g., Olapade and Leff 2004, McNamara and Leff 2004a, McNamara and Leff 2004b). The riparian vegetation is predominately hardwoods: sycamore, maple and oak, with approximately 90% canopy coverage during summer months.

Sample Collection and Preservation

Replicate (N=6), heterogeneous, surface sediment samples were collected on 8 September 2003, 7 November 2003, 28 February 2004 and 11 May 2004. Replicate (N=3 for summer and fall, N=6 for winter and spring) cobbles of a size large enough to scrape a 5 x 10 cm area for analysis were also collected on each sampling date. Samples were placed in Whirl-Paks® (Nasco, WI) and returned to the lab for fractionation of the sediments, preservation and analysis.

Substrate samples collected from the study reach were manually sieved gently under water, using U. S. Standard Sieves to sort the sediments into 3 size classes: large (≥ 4 mm < 8 mm), medium (≥ 1.18 mm < 4 mm), small (≥ 0.125 mm< 0.85 mm). A 5 cm x
10 cm area on each cobble was scraped gently with an acid washed and rinsed
toothbrush. All samples (both sediments and cobbles) were preserved with phosphate
buffered, 8% paraformaldehyde. Additionally, sodium pyrophosphate was added to
sediment samples that were then sonicated for 5 minutes to help dislodge bacterial cells
from the particles (Branson 2210 Sonicator, Danbury, CT). Samples were stored at 4°C
until analyzed for bacterial abundance.

Supporting physical and chemical data were measured on each date. Temperature
and pH were measured with an Oakton pH/mV/°C Meter and conductivity was measured
with an Oakton TDS/Conductivity/°C Meter (Con 10 Series). Turbidity was measured
with a Hach 2100P Turbidimeter (46500-00) while nitrate/nitrite-nitrogen and SRP were
measured with Hach kits, DR100 Colorimeter Nitrate (Low Range) (41100-13) and Hach
DR 100 Colorimeter Phosphorous, Reactive (41100-16), respectively.

A portion of each sediment sample was used to determine dry weight and ash-free
dry mass (AFDM). Dry weight was determined by drying over night at 104°C (Baxter
TempCon Oven, Scientific Products). AFDM was determined by placing dried samples
in a muffle furnace (Thermolyne 30400 Furnace) for 5 hours at 550 °C after which they
were reweighed.

Bacterial Abundance

Total bacterial abundance was determined via epifluorescent microscopy using
DAPI stain (Porter and Feig 1980). Samples were concentrated on black 0.2 μm
polycarbonate filters (Poretics, Livermore, CA), stained with DAPI (3-4 minutes), and rinsed using sterile, deionized, filtered water.

In FISH, rRNA within intact cells is hybridized with taxon-specific fluorescently labeled oligonucleotide probes (Saylor and Layton 1990). The taxon-specific probes used in this study targeted the Domain Bacteria (Amman 1990), the α-Proteobacteria and γ-Proteobacteria (Wagner et al. 1993), and the Cytophaga-Flavobacterium cluster (Amman et al. 1995). Species-specific probes used were Burkholderia cepacia (Braun-Howland et al. 1993), and Acinetobacter calcoaceticus (Wagner et al. 1993). FISH was accomplished using the protocol described by Lemke et al. (1997). Probe sequences and hybridization conditions are described in Olapade and Leff (2004).

Statistical Analyses

Statistical analyses were performed using two-way analyses of variance (ANOVA) to compare sediments of different sizes and sampling dates using SAS (SAS Institute Inc., Cary, NC, 1999). A one-way ANOVA was performed for cobble samples to examine differences among dates. Where appropriate (p ≤ 0.05), Tukey’s tests were performed to elucidate specific differences among the substrata sizes and sampling dates.
Results

Physical and Chemical Variables

The organic matter content of the sediments varied among size classes and seasons and in some cases was negligible (Table 1). Temperature varied over the four sampling dates from a low of 0.8 °C in February to a high of 17.6 °C in May 9 (Table 2). Flow rate was highest during the winter sampling date (0.65 m/s) although peak turbidity was during May (16.47 NTU). Inorganic nutrients varied over the four seasons, more so with the nitrate/nitrite than the SRP.

Bacterial Abundance on Sediments

For total bacterial counts (based on DAPI staining), there were significant differences among seasons, size classes and interaction effects \( p = 0.003, < 0.0001, \) and \( p = 0.020 \) respectively, Figure 1A). Fall abundance was significantly different from winter and spring and the smallest size category was higher than the other two size categories.

For the Domain \emph{Bacteria} there were also significant differences among seasons, size fractions and interaction effects \( p < 0.0001 \) for each, Figure 1B). In this case, spring abundance was higher than all other seasons. As with total counts, the smallest size category was higher than the medium and the large on some dates. There were no
differences between the medium and large sizes for either DAPI or Domain *Bacteria* abundance.

Abundance of the *α-Proteobacteria* differed significantly among sediment size classes and sampling dates (p < 0.0001 for both) and there was an interaction effect (p = 0.002, Figure 2A). Winter and spring abundance were higher than both fall and summer abundance. There was also a difference between abundance in summer and fall, with summer exhibiting much lower numbers than found during the other seasons. As with total abundance, there was a significant difference between the small size category (which exhibited the greatest numbers overall) and the other two categories, but the medium and large categories were not different from each other.

The *γ-Proteobacteria* differed significantly among seasons, size classes and there was a significant season*size class interaction (p < 0.0001, p = 0.010, and p = 0.015 respectively, Figure 2B). Abundance in summer and fall were higher than winter and spring abundance. As with the other bacteriological variables, abundance on the smallest size class tended to be greatest.

For the *Cytophaga-Flavobacterium* there were also significant differences among seasons, sizes and interaction effects (p < 0.0001, p = 0.030, and p = 0.040 respectively, Figure 2C). As with the *γ-Proteobacteria*, summer and fall abundance was higher than that found in winter and spring. For this taxon, there was a significant difference between the smallest and the largest size categories.

At the species level, *B. cepacia* exhibited a pattern unlike all the other taxa examined. While there were significant differences among seasons there were no
significant differences among sizes nor were there significant interaction effects \((p = 0.030, p > 0.05\) respectively Figure 3A). The only seasonal difference was between summer and spring.

The other species evaluated, *A. calcoaceticus*, also differed in abundance among seasons \((p < 0.0001, \text{Figure 3B})\). There were significant differences between sizes and an interaction effect \((p = 0.0002, \text{and } p = 0.002\) respectively). Winter and spring abundance, while not different from each other, were lower than summer and fall. Summer and fall were also different from each other. Highest numbers were on the smallest size particles.

Overall, when bacterial abundance was compared across sampling dates there were significant differences among seasons in all instances, no matter which taxon was examined. There were also generally differences among size classes.

*Bacterial Abundance on Cobbles*

On the cobbles, total bacterial numbers did not exhibit significant differences among seasons (Figure 4A). For the Domain *Bacteria*, on the other hand, there was a significant change with highest numbers in spring \((p < 0.0001, \text{Figure 4B})\).

For both *α-Proteobacteria* and *γ-Proteobacteria* there were significant differences among seasons. However, the patterns of difference were different, with peak abundance in spring for the *α-Proteobacteria* and peak abundance for the *γ-Proteobacteria* in summer \((p < 0.0001, \text{Figure 5A and B})\). *Cytophaga-Flavobacterium* abundance was also significantly different among seasons and exhibited a pattern similar to the *γ*-Proteobacteria \((p = 0.015, \text{Figure 5C})\).
The two species examined also exhibited significant seasonal differences. For *B. cepacia*, abundance was much higher in summer and fall than in winter and spring (p = 0.013, Figure 6A). The abundance of *A. calcoaceticus* was higher in summer than in the other seasons (p = 0.004, Figure 6B).

**Discussion**

Although for each taxon examined there were seasonal differences in abundance, the pattern of differences varied. In sediments, some taxa exhibited peak abundance in spring (Domain *Bacteria, α-Proteobacteria*) and others peaked in summer (*B. cepacia* and *A. calcoaceticus*). The *γ-Proteobacteria* and the *Cytophagae-Flavobacteria* peaked in the fall. Generally, the patterns of seasonal change were similar between sediments of different sizes and the cobbles suggesting that whatever factor(s) cause these differences among dates have similar impacts on communities throughout the sediments.

Seasonal variations in this study yielded different patterns than those reported in another study in the same stream that focused on bacterial communities on cobbles (Olapade and Leff 2004). In both cases, seasonal variations were observed, but Olapade and Leff (2004) found that, for some taxa, peaks in abundance were in seasons different from those found in the present study. For example, for the *Cytophaga-Flavobacterium*, abundance in the present study peaked in summer on sediments of all sizes, whereas Olapade and Leff (2004) found that abundance on cobbles peaked in winter.
In the time between the two studies, a significant flood event occurred that altered the geomorphology of the stream reach sampled. Thus, these discharge fluctuations, scouring, and geo-morphological changes, may have greatly influenced the microbial communities of the sediments. Moreover, temperatures on similar sampling dates were higher in 2003 (when samples were collected by Olapade and Leff, 2004) than 2004. This could alter the abundance or activity of cells (Bouvier and del Giorgio 2003) and because FISH targets rRNA, differences in ribosome content associated with varying activity may have resulted in differences in the number of cells detected (Poulsen et al. 1993). Temperature and discharge, as well as other factors, likely contribute to differences seen between the Olapade and Leff (2004) study and the current findings.

Differences among the three size categories of sediment did not yield the expected results and the hypothesis that there would be pronounced differences in community structure among the sediment size classes was not supported. The overall patterns of difference among size fractions for each taxon examined were similar in nearly all cases suggesting that the communities on the different fractions were highly similar.

According to Manz et al. (1999) initial colonization and attachment of bacteria are more likely to include bacteria from the $\beta$-Proteobacteria group first then $\alpha$-Proteobacteria and Cytophaga-Flavobacterium, which continue to dominate the mature biofilm. In the present study, the percentage that the Cytophaga-Flavobacterium contributed to the community was as high as 41% in the summer but only 0.04% in the spring on the cobbles and averaged 16% on the other sediments in the summer, and as with the cobbles, an even smaller fraction of a percent in the spring. This supports Manz
et al. (1999) with reference to the *Cytophaga-Flavobacterium* representing a greater percentage of mature biofilms that might be expected at the end of summer.

Overall, seasonal variations in abundance were predominant while differences among sediment sizes were of secondary importance. Typically, abundance was higher on the smallest particles and may result from a difference in surface area available for colonization. However, as reported in Santmire and Leff (next chapter) factors other than surface area contribute to differences among sediment grain sizes.
References


Olapade, O., Xueqing, G. and L. Leff. Abundance of three bacterial populations in selected streams, Microbial Ecology, in press.


<table>
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Table 1. Percent organic matter based on ash free dry mass of samples. Values are means and standard errors (N=6).
Table 2. Physical and chemical data for West Branch of the Mahoning River, September 2003 through May 2004. N=3 for all samples except AFDM where N=6. Values are means and standard errors (SE). The pH means and standard errors were calculated using the antilog values of each measurement and then converted back to pH values once calculated.

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<th>Conductivity (µS/cm)</th>
<th>Turbidity (NTU)</th>
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<td>483 (9)</td>
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Figure 1. Comparison of bacterial abundance over four seasons based on DAPI (A) and FISH using Domain *Bacteria* probe (B). Numbers are expressed per gram dry weight on log scale. Values represent means (N=6) and standard errors. Size classes are large (≥ 4 mm < 8 mm), medium (≥ 1.18 mm < 4 mm), and small (≥ 0.125 mm< 0.85 mm) .
Figure 2. Comparison of bacterial abundance over four seasons based on FISH using taxon-specific probes: A. \(\alpha\)-Proteobacteria B. \(\gamma\)-Proteobacteria C. Cytophaga-Flavobacterium. Numbers are expressed per gram dry weight on log scale. Values represent means (N=6) and standard errors. Size classes are large (\(\geq 4\ mm < 8\ mm\)), medium (\(\geq 1.18\ mm < 4\ mm\)), and small (\(\geq 0.125\ mm < 0.85\ mm\)).
Figure 3. Comparison of bacterial abundance over four seasons based on FISH using species-specific probes: *A. B. cepacia* B. *A. calcoaceticus*. Numbers are expressed per gram dry weight on log scale. Values represent means (N=6) and standard errors. Size classes are large (≥ 4 mm < 8 mm), medium (≥ 1.18 mm < 4 mm), and small (≥ 0.125 mm < 0.85 mm).
Figure 4. Comparison of bacterial abundance over four seasons based on DAPI (A) and FISH using Domain Bacteria probe (B). Numbers are expressed per cm² of cobbles on log scale. Values represent means (Summer and Fall N=3, Winter and Spring N=6).
Figure 5. Comparison of bacterial abundance over four seasons based on FISH using taxon-specific probes: A. α-Proteobacteria B. γ-Proteobacteria C. Cytophaga-Flavobacterium. Numbers are expressed per cm$^2$ of cobbles on log scale. Values represent means (Summer and Fall N=3, Winter and Spring N=6).
Figure 6. Comparison of bacterial abundance over four seasons based on FISH using species-specific probes: *A. B. cepacia* and *B. A. calcoaceticus*. Numbers are expressed per cm$^2$ of cobbles on log scale. Values represent means (Summer and Fall N=3, Winter and Spring N=6).
CHAPTER IV

The effect of sediment grain size on bacterial communities in streams

Abstract

Sediment bacteria are ubiquitous and important to the cycling of organic matter and nutrients in streams. Geomorphology and hydrodynamics provide the framework for streams and features like velocity, discharge, organic matter, and sediment composition help shape bacterial communities. In these experiments, impact of particle grain size on bacterial communities was investigated using artificial substrata of different sizes incubated in a Northeast Ohio (USA) stream. Abundance of specific bacterial taxa was examined using fluorescent in situ hybridization with which taxon-specific labeled oligonucleotides are hybridized with rRNA inside intact cells. Generally, there were differences in abundance among particles of different sizes but there were few differences among the suite of taxa examined. There were also few differences between substrata incubated in a pool or riffle in the stream or between those with or without organic matter amendment. Surface area effects represented one factor influencing bacterial abundance,

3To be submitted to the Journal of the North American Benthological Society by Judith A. Santmire and Laura G. Leff

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however, surface area alone did not account for the differences among the substrata indicating that other factors, such as permeability and porosity, are also important.

**Introduction**

There are important connections between sediments and associated bacteria in streams and rivers. Several researchers have studied metabolic activity in sediments (Cardinale et al. 2002, Sabater and Romani 1996, Bott and Kaplan 1985) and metabolic activity differs among sediments of different grain sizes (Romani and Sabater 2001). Overall, grain size can be an important factor determining bacterial abundance (Dong et al. 2002). Given that bacterial activity in sediments is vital to stream metabolism (Fischer and Pusch 2001), determining the relationship between bacterial communities and sediment grain size can provide insight into factors controlling bacteria in streams. Multiple factors affect biota in lotic ecosystems although it is difficult to determine what combinations of factors are most significant. Many researchers have documented the importance of organic matter to bacterial metabolism (Bott et al. 1984, Findlay and Sobczak 2000, Rier and Stevenson 2001) and allochthonous inputs from water column solutes are important resources for sediment communities (Chafiq et al. 1998).

How microorganisms utilize organic matter and nutrients is likely affected by the physical features of a stream reach (including sediment characteristics) and may also significantly impact bacterial community structure. The type of sediment and its grain
size (e.g. fine sand, cobbles) is related to velocity, geology and the morphology of the stream. The hydrodynamics of a lotic ecosystem may, in fact, be one of the greatest determinants of stream features, and the types of sediments that are present at a given location (Fischer et al. 2003). It may be as important, or even more important, than organic matter (Findlay and Sinsabaugh 1999).

To examine differences in bacterial communities that develop on varying size sediments, experiments were conducted using artificial substrata (glass beads and tiles) to help provide quantitative results. The combined use of artificial substrata and enumerating bacteria of different taxa using fluorescent in situ hybridization (Amann et al. 1990) made it possible to evaluate bacterial community structure in a field setting. In tandem with this approach, other related environmental features were investigated, i.e. the effect of organic matter amendment and the effect of stream location (pool versus riffle), to determine if these features work in concert, or independently, in the establishment of benthic bacterial communities.

Specifically, it was hypothesized that differences in communities on substrata of different sizes may be attributable to differences in surface area and/or packing of particles. Moreover, it was hypothesized that differences in organic matter retention and accumulation by sediments of different sizes, as well as differences in location within a stream (as tied to the distribution of sediments of different sizes in the field and related to water velocity), influences bacterial community structure.
Methods

Site Description

This experiment was conducted in a pool/riffle segment (30 meters long and 16 meters wide) of the West Branch of the Mahoning River, a 4th order stream within the Mahoning River watershed in Northeast Ohio (Latitude 41°09'41", Longitude 81°11'50"OH EPA Technical Report MAS/1995-12-14), USA. Average depth over the one month incubation was 19 cm for the riffle and 17 cm for the pool.

Experimental Design

A preliminary study was conducted for one month within the experimental reach, commencing 20 July 2003, to determine if artificial substrata (i.e., glass beads and unglazed tiles) could be used to simulate natural substrate. The advantages of using artificial substrata include their uniform size and composition as well as the ability to calculate surface areas of these materials. Natural substrata, collected from the experimental reach, were sieved manually using U. S. Standard Sieves, then acid washed and rinsed several times prior to placing into packets (as described below), as were the artificial substrata. Packets containing natural or artificial substrate were incubated at the study site and retrieved after one month. Total bacterial numbers were not significantly different between the natural and artificial substrata (data not shown) and subsequently the artificial substrata were used in the experiments. Furthermore, this experiment illustrates that bacteria were effectively able to colonize the substrata in the packets, as
the abundance of cells in the packets was comparable to natural abundances in the field (Santmire and Leff, in review).

Three sizes (diameters: 0.1 mm, 1.0 mm, & 5.0 mm) of soda lime glass beads (0.1 & 1.0 mm: Biospec; 5.0 mm: Fisher) and unglazed tiles 5x5 cm were used as artificial substrata. Packets were made of 80 µm mesh (Nitex® Brand) small aperture nylon plankton netting (Wildco Wildlife Supply Company, MI), cut into squares (20x20 cm). Each square was filled with 50 g of one size bead (e.g. 0.1 mm diameter), the points were gathered together, and tied tightly at the top with cable ties. To create packets for the tiles, four unglazed tiles were placed in the same netting cut into 2 squares and sewn together, then fastened with a cable tie. Once the packets were made they were attached to tent stakes (4 per tent stake, one of each size category) for deployment in the stream reach. Packets for experiments described below were deployed on 5 November 2003. Physical and chemical variables were taken the week of placement (7 November), at the midpoint of incubation (21 November) and upon retrieval of packets (8 December).

To examine the potential impacts of differing water velocity, replicate packets (N=4 for each size class) were placed in two sites in the stream. An adjacent pool and riffle were chosen for placement of the packets with an average velocity of 27 cm/second in the riffle and 0.79 cm/second in the pool.

Because organic matter accumulation may vary among sediments of different sizes, the potential role of organic matter addition on bacterial communities was examined on the varying sized substrata. In this experiment, 1.5 g of freshly collected, senescent riparian, sugar maple leaves (air dried overnight) was placed in a non-organic
empty tea bag (In Pursuit of Tea, Inc., New York) within each plankton netting packet (control contained an empty tea bag placed within the packet). Four replicates of each size class were used for the control and the amended treatments and the packets were incubated in the same riffle as the flow rate/placement experiment (downstream of those packets, to prevent effects from packets with organic matter additions).

Subsequently, an additional experiment was performed to determine whether the packing of beads (considering the possibility that the amount of artificial substrate in each packet produced a response to colonization that would vary if there were less mass per packet) in the experimental packets impacted the bacterial communities. Two of the bead sizes were chosen (0.1 mm and 5.0 mm diameter); the same packet design and incubation time were used but varying amounts of beads were added (15, 25, and 50 g) per packet (N=5 for each amount of beads). Packets were deployed 21 June 2004 and retrieved 20 July 2004.

Sample Collection and Preservation

Packets were retrieved after one month, returned to the lab and samples from each packet were preserved with phosphate buffered, 8% paraformaldehyde. Sodium pyrophosphate was added to the samples and then they were sonicated for 5 minutes (Branson 2210 Sonicator, Danbury, CT) and stored at 4° C. One set of organic matter samples (amended) was lost and reduced the number of amended samples in that experiment to N=3.
At the start, midpoint and end of the experiment temperature and pH were measured with an Oakton pH/mV/°C Meter and conductivity was measured with an Oakton TDS/Conductivity/°C Meter (Con 10 Series). Turbidity was measured with a Hach 2100P Turbidimeter (46500-00), while nitrate/nitrite-nitrogen and soluble reactive phosphorus were measured with Hach kits, DR100 Colorimeter Nitrate (Low Range) (41100-13) and Hach DR 100 Colorimeter Phosphorous, Reactive (41100-16), respectively. A portion of each packet was used to determine dry weight. Dry weight was determined by drying over night at 104°C (Baxter TempCon Oven, Scientific Products) and then samples were weighed again.

**Bacterial Abundance**

Total numbers of bacteria were determined via epifluorescent microscopy using DAPI stain (Porter and Feig 1980). Samples were concentrated on black 0.2 µm polycarbonate filters (Poretics, Livermore, CA), stained with DAPI (3-4 minutes), and rinsed using sterile, deionized, filtered water.

In FISH, cellular rRNA is hybridized with taxon-specific fluorescently labeled probes (Saylor and Layton 1990). The probes used for this experiment targeted the Domain Bacteria (Amman 1990), the Cytophaga-Flavobacterium cluster (Amman et al. 1995), the Proteobacteria (α & γ) (Wagner et al. 1993), Burkholderia cepacia (Braun-Howland et al. 1993), and Acinetobacter calcoaceticus (Wagner et al. 1993). Probe sequences and hybridization conditions are described in Olapade and Leff (2004). FISH was accomplished as in Lemke et al. (1997).
Data Analyses

Bacterial abundance determined as described above was expressed in two ways: per gram and per cm\(^2\) of surface area (abundance on tiles was expressed only on a surface area basis). Although it is convenient for comparison to natural substrate, solely expressing numbers per g does not necessarily represent the most biological relevant factor related to differences among particles of different sizes and is not useful for comparing abundance on tiles (which are comparatively heavy) to those on the beads. Thus, the surface area of artificial substrata in the packets was calculated for each sample. To determine the surface area of the glass beads the following calculations were performed. For tiles, the surface area was 100 cm\(^2\).

1. Total volume of sample in cm\(^3\) (where density of glass beads = 2.65 g/cm\(^3\)) = Total wt of sample in g / density in g/cm\(^3\)
2. Volume of one sphere in cm\(^3\) (where \(r = \) radius of a bead) = \(\frac{4}{3} \pi r^3\)
3. Number of spheres per packet = Total volume of sample / volume of one sphere
4. Surface area of one sphere = \(4\pi r^2\)
5. Surface area of all beads in a packet in cm\(^2\) = surface area of one sphere times number of spheres in a packet

Statistical Analyses

Two-way analyses of variance (ANOVA) were performed for each experiment (pool versus riffle, organic matter amendment, packing) using SAS (SAS Institute Inc.,
Cary, NC, 1999). Where appropriate (p \leq 0.05), Tukey’s tests were performed to elucidate specific differences among the different size artificial substrata.

Results

Physical and Chemical Variables

Temperature of the experimental reach declined over the course of the month from 9.6°C the week of deployment, to 0.8°C the morning they were retrieved (Table 1). The mean pH was relatively stable in November, but was higher in December. Conductivity showed much more temporal variation with the highest conductivity noted in December.

Pool and Riffle Comparison

When overall numbers of bacteria were expressed per gram dry weight of substrate, abundance on the 5.0 mm beads was significantly lower than on the 0.1 mm and 1.0 mm beads, for both DAPI (p = 0.009) and Domain Bacteria counts (p = 0.002, Figure 1A and 1B). Overall, total abundance was highest on the smallest beads and lowest on the largest beads. There were no significant differences between pool and riffle-incubated packets, for either bacteriological variable, nor were there any interaction effects between substrate size and stream location.
Significant differences among artificial substrata of different sizes were also noted when expressing the results as numbers of bacteria per cm² (p < 0.0001, Figure 1C). For the Domain Bacteria, there were significant differences between the 0.1 mm and 5.0 mm beads and also both the 0.1 mm and the 5.0 mm beads were different than the 1.0 mm beads and tiles (p < 0.0001, Figure 1D). For both DAPI and Domain Bacteria numbers, the 5.0 mm riffle-incubated beads had the highest numbers and the 0.1 mm beads had the lowest. There were also significant differences between locations (pool versus riffle) for DAPI stained cells (p = 0.012) on the 5.0 mm beads, but this was not the case for any of the substrate size classes for the Domain Bacteria.

When examining the Proteobacteria on a gram dry weight basis, the α-Proteobacteria were significantly different in abundance between the 0.1 mm and 5.0 mm beads (p = 0.022), with the highest numbers on the 0.1 mm beads and lowest on the 5.0 mm beads (Figure 2A). For the α-Proteobacteria there were no significant interactions between substrate size and location within the stream reach. Only significant interaction effects were detected for the γ-Proteobacteria (p = 0.014, Figure 2B).

Significant differences were also detected for the Proteobacteria when the results were expressed as numbers per cm² (Figure 3). The numbers of α-Proteobacteria on the 5.0 mm beads were significantly higher than on any of the other sizes (p < 0.0001, Figure 3A). Interestingly, when abundance of the γ-Proteobacteria was expressed per cm² there were significant differences among sizes, location (pool versus riffle) and interaction effects (p <0.0001, p = 0.003, and p = 0.0002 respectively, Figure 3B). For bacterial
abundance reported per cm², the highest numbers for \( \gamma \)-Proteobacteria were on the 5.0 mm beads and the lowest were on the 0.1 mm beads.

Members of the *Cytophaga-Flavobacterium* cluster clearly exhibited a response to substrate size \((p = 0.001)\) and pool versus riffle placement \((p = 0.032)\). Abundance on the 1.0 mm beads was significantly different from the 0.1 mm and 5.0 mm beads (Figure 2C). Contrary to the other bacteriological variables, highest numbers were on the 1.0 mm riffle-incubated beads.

The *Cytophaga-Flavobacterium* cluster, when expressed per cm², exhibited significant differences based on both size of substrate and location (pool versus riffle) of incubation effects (Figure 3C). There were significant differences when comparing the 1.0 and 5.0 mm beads (that had the highest numbers) to the 0.1 mm beads (that had the lowest numbers) and the tiles \((p < 0.0001 \text{ for size, } p = 0.26 \text{ incubation effects})\).

When the two specific species were considered on a per gram basis, abundance of *B. cepacia* was highest on the 0.1 mm beads and lowest on the 5.0 mm beads \((p = 0.004, \text{ Figure 3D})\). Additionally, there was a significant interaction effect between size and location of incubation \((p = 0.009)\). For *A. calcoaceticus*, there were no significant differences (Figure 3E).

On a surface area basis, *B. cepacia* was significantly different in abundance on the tiles and the 0.1 mm beads compared to the 1.0 and 5.0 mm beads \((p = 0.012)\) and there was a significant interaction effect \((p = 0.004, \text{ Figure 3D})\). In this instance, highest numbers were on the tiles while lowest numbers were on the 0.1 mm beads. For *A. calcoaceticus*, there were significant differences among particle sizes \((p < 0.0001)\) but
there were no location or interaction effects (Figure 3E). Both the 0.1 mm beads and tiles were different compared to the 1.0 and 5.0 mm beads. As with the other species, *B. cepacia*, the highest numbers were on the tiles and the lowest were on the 0.1 mm beads.

**Organic Matter Addition**

In the organic matter amendment experiment, when expressed as per gram dry weight of sample, DAPI-stained bacteria (p = 0.012) were significantly different between 0.1 mm and 5.0 mm beads, but Domain *Bacteria* counts (p = 0.229) were not significantly different between substrata of different sizes (Figure 4 A and B). There were no significant differences between controls and amended packets nor were there any interaction effects for either DAPI or Domain *Bacteria*.

When abundance was expressed on a per cm$^2$ basis, both DAPI (p = 0.002) and Domain *Bacteria* (p = 0.001) counts were significantly different among substrate sizes (Figure 4 C and D). For both bacteriological variables, the 5.0 mm beads were different from all other sizes. Like the pool versus riffle comparison, in each instance, the 5.0 mm beads had the highest abundance and the 0.1 mm beads had the lowest. As with the abundance expressed on a per gram basis, for both DAPI and Domain *Bacteria*, there were no significant effects for treatment (control versus organic matter addition) nor were there any interaction effects.

For *α-Proteobacteria* abundance, expressed per gram dry weight, there were significant differences among sizes (p = 0.039), between the control and organic matter
amendment (p = 0.039), and a significant interaction (p = 0.05, Figure 5A). Abundance of the \textit{\alpha-Proteobacteria} was highest on 0.1 mm beads and lowest on 5.0 mm beads. For the \textit{\gamma-Proteobacteria} abundance differed significantly only among substrate sizes (p = 0.05); abundance was highest on 0.1 mm beads and lowest on 5.0 mm beads (Figure 5B).

When \textit{\alpha-Proteobacteria} numbers were expressed per cm$^2$, there were significant differences only among sizes (p = 0.014) with the 5.0 mm size beads different from all other sizes; lowest abundance was on the 0.1 mm beads (Figure 6A). The \textit{\gamma-Proteobacteria} exhibited significant differences among sizes (p < 0.0001), between the controls and organic matter amendments (p = 0.045), and there were interaction effects (p = 0.002). The 5.0 mm beads differed from all other size classes and had the highest numbers while the lowest numbers were on the 0.1 mm beads (Figure 6B).

On a per gram basis, abundance of \textit{Cytophaga-Flavobacterium} was significantly different among sizes (p = 0.0002) and with organic matter amendment (p=0.003; Figure 5C). Highest numbers were on the 1.0 mm beads while the 5.0 mm beads had the lowest. On a per surface area basis, numbers of \textit{Cytophaga-Flavobacterium} differed significantly among sizes (p < 0.0001) with 0.1 mm beads (which had the lowest numbers) different from both 1.0 and 5.0mm beads (which had the highest numbers). The 1.0 and 5.0 mm beads were also different from the tiles. There were no significant differences between treatments (organic matter addition versus control) nor were there any interaction effects (Figure 6C).

At the species level, when expressed on a per gram basis, there were no significant differences between amended and control packets or substrata of different
sizes. On a surface area basis, no significant differences were observed for *B. cepacia* (Figure 6D). The *A. calcoaceticus* abundance differed among sizes (*p* = 0.004) when comparing 5.0 mm beads (that had the highest numbers) to the 0.1 (that had the lowest numbers) and 1.0 mm beads (Figure 6E).

**Packing Experiment**

Total bacterial abundance (based on DAPI staining) supported the premise that substrate grain size had a significant impact (*p* < 0.0001) and that packing, or density of beads in the experimental packet, did not, since neither treatment (i.e., differences among packets with varying amounts of beads) nor interaction effects were significant (Figure 7A). The Domain **Bacteria** presented a different picture with significant differences observed among substrate sizes (*p* < 0.0001), density of beads (*p* = 0.002) and interaction effects (*p* = 0.006, Figure 7B).

At the taxon-specific level, **α-Proteobacteria** abundance differed significantly between substrates of the two sizes (*p* < 0.0001) and among packets with different bead densities (*p* = 0.0003; Figure 8A). As with the Domain **Bacteria**, there were interaction effects (*p* = 0.0004). The **γ-Proteobacteria** responded similarly to the **α-Proteobacteria** with both significant size (*p* < 0.0001) and density (*p* = 0.0504) effects (Figure 8B). Unlike the **α-Proteobacteria**, there were no interaction effects.

**Cytophaga-Flavobacterium** exhibited significant differences among sizes (*p* < 0.0001), with no other significant effects (Figure 8C). For both *B. cepacia* and *A.
calcoaceticus, differences among sizes were significant but packing effects were not (p = 0.0001 and p < 0.0001, Figure 8D and 8E, respectively).

**Discussion**

The original hypothesis, that differences in substrate size would affect bacterial abundance, was substantiated, with the largest differences generally occurring between the 0.1 mm beads and the 5.0 mm beads, with only a few exceptions. Location of incubation (riffle versus pool) and organic matter amendments were not as clearly important to all the taxa examined.

The size of the substrata with the highest abundance varied among taxa to some degree, although responses of the different bacterial groups were generally similar perhaps in relation to the amount of surface area available. Yet, surface area alone does not explain the differences among the substrate sizes. When bacterial numbers were expressed on a surface area basis there were still large differences among substrates of different sizes indicating that sediment grain size effects on bacteria involve more than just differences in surface area.

One might expect that increased surface provides more colonization surface for bacteria and thus speculate that the primary differences among substrates are attributable to different surface areas. Although it is difficult to elucidate the array of associated factors that contribute to the large differences among substrates of different sizes, there
are several other factors that may have caused the differences among substrata.
Specifically, porosity and permeability are an important consideration in sediments.

Because of the nature of the experiments, one might expect decreased
permeability of finer sediments that may alter bacterial access to the surfaces. The fact
that the different taxa examined were generally similar in their responses reinforces the
possibility that differences among substrate sizes are due to some physical phenomenon.

Generally, the addition of organic matter to the packets had little impact on the
abundance of bacteria. The occurrence of dissolved organic matter (DOM) in lotic
sediments is well documented (Wetzel 1984), as is the importance of bacterial uptake of
Although the leaves in the packets were leaching out DOM, possibly this material was
too recalcitrant to not be utilized effectively by the bacteria or perhaps the surrounding
water column provided sufficient DOM for the cells such that supplementing this amount
had no effect (Ostrofsky 1997).

The patchiness of lotic ecosystems, importance of flow rate, and heterogeneity of
substrates is well documented (Cardinale et al. 2002). For these reasons, it was
hypothesized that there would be differences between the pool and riffle. For the most
part this was not the case. Perhaps any differences among sediments in the field are
related to the size of the particles themselves rather than water column effects. Thus, it is
likely that there will be differences between natural bacterial communities on substrata of
different sizes but this may greatly depend on temporal and spatial heterogeneity at the
micro-scale (given that in our experiments sediments compared were very homogeneous and single sizes of particles were incubated together).

Differences among substrate sizes were in part related to surface area but based on the data analyzed, there are additional factors that must be considered. The packing experiment reveals that this difference is not completely attributable to differences in packing of the different beads either (or permeability of the groups of beads), but the packets were homogeneous. Perhaps it relates to the physical and chemical properties of the interstitial spaces, and because of homogeneity, permeability was not really an issue in these experiments. In addition, biotic interactions may differ on substrates of different sizes, for example, access of protozoa, meiofauna or other bacterial consumers, may differ substantially with sediment grain size (Hamels et al. 2001).

It is important also to consider the suite of taxa evaluated for these experiments in relation to the patterns that emerged. Large taxonomic groups, e.g., the Domain *Bacteria*, *Proteobacteria*, and *Cytophaga-Flavobacterium*, while seeming to exhibit similar overall patterns, might exhibit different patterns if specific species were examined in each group. For example, *A. calcoaceticus* (a member of the *γ-Proteobacteria*) exhibited responses that differed from those of the *γ-Proteobacteria* as a whole.

As with other studies, results can vary among similar environments within the same system. In the Olapade and Leff study (2004), current results differed, using the same FISH probes on cobbles, in the same stream. Romani and Sabater (2001) report lower biomass on sand but increased efficiency of enzymatic activity. Marxsen (2001) also reports clear differences in bacterial production on fine versus coarse substrate. The
differences among substrate sizes are clearly an important factor influencing bacteria in streams and substrate impacts involve more than differences in surface area.
References


Table 1. Physical and chemical variables during incubation of experimental packets with means and (standard error).

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<th>pH</th>
<th>Conductivity (µS)</th>
<th>Velocity (cm/s)</th>
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<td>7.5 (8.7)</td>
<td>654.3 (0.9)</td>
<td>20.0 (11.5)</td>
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<tr>
<td>11/21/03</td>
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<td>7.3 (9.4)</td>
<td>459.0 (67.0)</td>
<td>37.7 (14.3)</td>
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<tr>
<td>12/8/03</td>
<td>0.8 (0.0)</td>
<td>8.1 (9.1)</td>
<td>979.7 (3.7)</td>
<td>16.6 (7.6)</td>
</tr>
</tbody>
</table>
Figure 1. Comparison of bacterial abundance on beads incubated in a pool versus a riffle based on DAPI (A and C) and FISH using Domain *Bacteria* probe (B and D). Numbers are expressed per gram artificial substrate (A and B) and per cm$^2$ of artificial substrate (C and D). Values represent means (N=4) and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
Figure 2. Comparison of bacterial abundance on beads incubated in a pool versus a riffle expressed per gram of artificial substrate based on FISH using taxon (A, B, and C) and species-specific (D and E) probes: A. α-Proteobacteria B. γ-Proteobacteria C. Cytophaga-Flavobacterium D. B. cepacia E. A. calcoaceticus. Values represent means (N=4) and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
Figure 3. Comparison of bacterial abundance on beads incubated in a pool versus a riffle expressed per cm² of surface area of artificial substrate based on FISH using taxon (A, B and C) and species-specific (D and E) probes: A. α-Proteobacteria B. γ-Proteobacteria C. Cytophaga-Flavobacterium D. B. cepacia E. A. calcoaceticus. Values represent means (N=4) and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
Figure 4. Comparison of bacterial abundance in packets amended with organic matter versus those with no added organic matter based on DAPI (A and C) and FISH using Domain I probe (B and D). Numbers are expressed as per gram artificial substrate (A and B) and per cm² of artificial substrate (C and D). Due to lost sample in situ, N=3 for amended, and N=4 for control and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
Figure 5. Comparison of bacterial abundance (per gram of artificial substrate) in packets amended with organic matter versus those with no added organic matter based on FISH using taxon (A, B and C) and species (D and E) specific probes: A. *α-Proteobacteria* B. *γ-Proteobacteria* C. Cytphaga-Flavobacterium D. *B. cepacia* E. *A. calcoaceticus*. Due to lost sample in situ, N=3 for amended, and N=4 for control and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
Figure 6. Comparison of bacterial abundance (per cm$^2$ of surface area of artificial substrate) in packets amended with organic matter versus those with no added organic matter based on FISH using taxon (A, B, and C) and species-specific (D and E) probes:

A. $\alpha$-Proteobacteria  B. $\gamma$-Proteobacteria  C. Cytophaga-Flavobacterium  D. B. cepacia  E. A. calcoaceticus. Due to lost sample in situ, N=3 for amended, and N=4 for control.

Values are means and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
Figure 7. Bacterial abundance expressed in per cm² surface area of artificial substrate for packing experiment using DAPI (A) and Domain *Bacteria* (B). Values are means (N=5) and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
Figure 8. A. Bacterial abundance expressed in per cm$^2$ surface area of artificial substrate for packing experiment using FISH taxon-specific (A, B, and C) and species-specific (D and E) probes: A. α-Proteobacteria B. γ-Proteobacteria C. Cytophaga-Flavobacterium D. B. cepacia E. A. calcoaceticus. Values are means (N=5) and standard errors. Note: values are on a log scale and the y-axis scale may vary depending on the magnitude of the abundance.
CHAPTER V

Sediments and bacterial communities in streams: Do bacterial communities from differing grain size sediments differ in their ability to colonize particles of different sizes?4

Abstract

The sediments of lotic ecosystems are important sites for bacterial growth and a repository of nutrients and organic matter that can be cycled by microorganisms. Grain size of substrate can affect the abundance of bacteria and, in this experiment, the role of grain size in determining bacterial colonization was examined. To this end, experimental microcosms were used to test the hypothesis that bacterial communities developed in situ in a Northeast Ohio stream on two sizes of artificial substrate (glass beads 0.1 and 5.0 mm in diameter) would exhibit differential response when inoculated onto glass beads of the other size in laboratory microcosms. Microcosms were set up in the dark using 1-L beakers containing filtered, autoclaved stream water and glass beads. Bacteria recovered from beads incubated in the study stream were standardized to similar abundances before inoculation into microcosms. Controls were inoculated with bacteria from the same size beads and treatments consisted of either 0.1 mm communities inoculated on to 5 mm

4To be submitted to Oikos by Judith A. Santmire and Laura G. Leff
beads or 5 mm communities inoculated on to 0.1 mm beads. Samples were collected at the start of the experiment and after 24, 48, and 144 hours. On each date, nitrate-nitrite nitrogen and reactive phosphorous were measured using unfiltered water samples from each microcosm. There were no significant differences for the nitrogen nor were there any significant differences for the reactive phosphorus. There were significant differences on both the large and small bead microcosms on day 6 for $\alpha$-Proteobacteria and also on day 6 for the large beads for the *Burkholderia cepacia*. There was also a significant difference on day 2 for *Acinetobacter calcoaceticus*. In all instances the bacteria were more abundant on beads of the same size they had colonized on in the stream, than those colonizing in the microcosm on the different sized substrate. Both the *B. cepacia* (part of $\beta$-Proteobacteria) and the $\alpha$-Proteobacteria are known to be early colonizers of biofilms and may have outcompeted other bacteria for limited available nutrients during the course of the experiment.
Introduction

Attempting to characterize bacterial species in streams and rivers has long been complicated by the low culturability of environmental microorganisms (McDougald et al. 1998). Now with molecular methods, such as fluorescent in situ hybridization, it is possible to meet some of these challenges (Amann et al. 1990).

Research reported in earlier chapters demonstrated that substrate particle size can influence bacterial abundance and that there are some differences in the community composition on sediments of different grain sizes. This raises the question: does the substrate size itself, help define the type of bacterial species or taxa that will colonize a given particle size? One approach to test this idea is the use of laboratory microcosms (Wagner-Doebler et al. 1992) and the artificial substrata used for the experiments in Chapter IV.

The purpose of this experiment was to see if a bacterial community originating from one size of artificial substrate would respond differently if introduced to a different size substrate. Specifically, based on the data in Chapter IV, the 0.1 mm and 5.0 mm beads were chosen to evaluate their responses under these experimental conditions. Thus, bacteria colonized in the stream on 5.0 mm beads were inoculated onto 0.1 mm beads in a microcosm. Conversely, bacteria colonized in situ on 0.1 mm beads were used to colonize 5.0 mm beads in a microcosm. I hypothesized that bacterial communities colonized on one particle size in situ (either 0.1 mm or 5.0 mm diameter) would respond differently (via reduced or increased abundance) as a direct effect, if introduced to
particles of a different size (0.1>5.0 and 5.0>0.1) Furthermore, to test differences, half the microcosms were inoculated with bacteria incubated on the same size beads in the stream (0.1>0.1 and 5.0>5.0).

**Methods**

*Substrate Colonization Site and Experimental Design*

Artificial substrata were incubated in mesh packets for one month (deployed on 18 August 2004 and retrieved on 18 September 2004) in the West Branch of the Mahoning River. The West Branch of the Mahoning River is a 4th order stream within the Mahoning River watershed in Northeast Ohio (Latitude 41°09'41", Longitude 81°11'50"OH EPA Technical Report MAS/1995-12-14). Two sizes (diameters: 0.1 mm, and 5.0 mm) of soda lime glass beads (0.1: Biospec; 5.0 mm: Fisher) were used as artificial substrata. Packets were made of 80 µm mesh (Nitex® Brand) small aperture nylon plankton netting (Wildco Wildlife Supply Company, MI), cut into squares (20x20 cm). Each square was filled with 50 g of one size bead, the points were gathered together and tied tightly near the top with cable ties. Once the packets were made they were attached to tent stakes (2 per tent stake, one of each size) for deployment in the stream.

Immediately upon retrieval approximately 10 grams of the stream-incubated beads were weighed and cells were stained with DAPI to determine abundance of cells in each sample. Amounts of sample for seeding of microcosms were adjusted to ensure the
same relative numbers of bacteria were added to each experimental microcosm (a final number of $1.34 \times 10^6$ cells per mL). Samples needing to be adjusted were diluted with appropriate amounts of autoclaved, filtered stream water.

**Microcosm Experiment**

Twelve one L beakers containing 100 grams of beads per beaker (6 with 0.1 mm diameter beads and 6 with 5.0 mm diameter beads) were filled with 600 mL of sterilized stream water. A Phipps and Bird Stirrer assembly (Model 7790-400, Richmond, VA) was used to circulate water in the microcosms and was set at 30 RPM. The assembly was kept in the dark. Each beaker was seeded with a predetermined amount of bacteria taken from beads that had been incubated in situ for one month. Six of the beakers (3 of each size) were seeded with bacteria from the same sized beads, and were used as controls. The other six beakers were seeded with stream-incubated beads of the opposite size (bacteria from 0.1 mm beads inoculated onto 5.0 mm microcosm substrate and bacteria from 5.0 mm beads inoculated onto 0.1 mm microcosm substrate).

Microcosms were sampled after 0 hours, 24 hours, 48 hours and 144 hours. All samples collected for bacterial enumeration were preserved in 30 mL of buffered, 8% paraformaldehyde and 10 mL of sodium pyrophosphate. All samples were kept at $4^\circ C$ until analyzed.

At the same time, water samples were analyzed for nitrate/nitrite-Nitrogen and Soluble Reactive Phosphorus (SRP) using a Latchat Instruments Quikchem 8000 Series Automated Ion Analyzer (Loveland, Colorado). Quikchem method 10-107-04-7-J, low
flow method was used for nitrate-nitrite nitrogen (0.10-10.0 mg N/L as nitrate and nitrite). For the orthophosphate, Quikchem method 10-115-01-1-B was used by flow injection (10-200 µg P/L). Both procedures used analysis colorimetry.

**Bacterial Abundance**

Total numbers of bacteria were determined via epifluorescent microscopy using DAPI stain (Porter and Feig 1980). Samples were concentrated on black 0.2 µm polycarbonate filters (Poretics, Livermore, CA), stained with DAPI (3-4 minutes), and rinsed using sterile, deionized, filtered water.

In FISH, cellular rRNA is hybridized with taxon-specific fluorescently labeled probes (Saylor and Layton 1990). The probes used for this experiment targeted the Domain *Bacteria* (Amman 1990), the *Cytophaga-Flavobacterium* cluster (Amman et al. 1995), the *Proteobacteria* (α & γ) (Wagner et al. 1993), *Burkholderia cepacia* (Braun-Howland et al. 1993), and *Acinetobacter calcoaceticus* (Wagner et al. 1993). Probe sequences and hybridization conditions are described in Olapade and Leff (2004). FISH was accomplished as in Lemke et al. (1997).

**Statistical Analyses**

Two-tailed unpaired t-tests were performed using Microsoft Excel to compare colonization of same size beads by two different treatments (colonized by bacteria on same size beads and colonized by bacteria on different sized beads).
Results

**Nitrogen and Phosphorus**

Nitrate/nitrite-nitrogen increased over the 6 days of the experiment with starting levels ranging from 0.35 mg/L to 0.83 mg/L on day 0 to a range of 0.76 mg/L to 1.1 mg/L on day 6 (Figure 1A). There was a less consistent pattern with SRP (Figure 1B). On day 0, the range was 15.3 µg/L to 29.5 µg/L. At day 2, there were varied responses with a decline in phosphate in 3 groups microcosms (11.3 to 15.0 µg/L) and a sharp increase in the SRP in one group of microcosms (40.3 µg/L) that stayed at the same level through day 6. Two of the other groups showed a slight increase (18.6 µg/L and 20.8 µg/L respectively) and the third group that had declined by day 2 continued to decline slightly to 8.9 µg/L of SRP.

**Bacterial Abundance**

Abundance based on enumeration via DAPI staining and FISH with the Domain *Bacteria* probe did not show any significant differences on any of the days of the experiment between either treatment (0.1 community on 5 mm beads, 5 community on 0.1 mm beads) and their corresponding controls (0.1 community on 0.1 mm beads, 5 community on 5 mm beads; Figure 2A and 2B). Responses were similar on each of the days regardless of the inoculant used in the microcosm.

For the *Proteobacteria* taxa examined, a significant difference was apparent between the treatment and the control on the small beads for the *α-Proteobacteria* (p =
There was also a significant difference on the large beads for the $\alpha$-Proteobacteria ($p = 0.03$). In each instance, the bacteria colonizing the microcosm beads seemed to colonize better when introduced to beads of the same size they had colonized on in the stream. There were no significant differences for either the $\gamma$-Proteobacteria or the Cytophaga-Flavobacterium (Figure 3B and 3C).

At the species level, there was a significant difference on day 2 for B. cepacia on the small beads ($p = 0.05$, Figure 4A). There was a significant difference on the A. calcoaceticus, on the 5.0 mm beads and, as with the $\gamma$-Proteobacteria, it occurred on day 6 ($p = 0.02$, Figure 4B). As with the $\alpha$-Proteobacteria there were higher counts on bacteria colonizing beads of the same size on which they had been incubated in the stream.

**Discussion**

The hypothesis that bacteria colonized on one size substrate would respond differently when introduced to a contrasting substrate was somewhat supported. According to Manz et al. (1999), the $\alpha$-Proteobacteria group, are some of the earlier colonizers that also remain well established in mature biofilms. Their response might be due in part to their competitive success early in colonization and maintain this level of abundance throughout the experiment.

B. cepacia, (a $\beta$-Proteobacteria) are known to be among the early colonizers of biofilm as well according to Manz (1999). This very diverse group of bacteria is
successful in a variety of conditions (Palleroni 1975). In each case where there were
significant differences, it appeared that the taxa were more abundant in the microcosm on
beads of the same diameter as those they had colonized on in the stream.

However, the \textit{A. calcoaceticus}, (a $\gamma$-\textit{Proteobacteria}) has been shown in other
studies to be difficult to grow in the laboratory, yet when colonized on similar sized
beads in the lab there was a significant effect after 6 days, compared to the other sized
beads on which they were inoculated (Lemke and Leff 1999).

The \textit{Cytophaga-Flavobacterium} are chemoorganotrophic and efficient at
degrading recalcitrant compounds (Kirchman 2002). This ability coupled with their
tendency to be later colonizers may be part of the reason no significant differences were
seen. Once again there are more factors involved than the substrate size alone. Limited
dissolved nutrients were present in the microcosms at the outset, but whatever
preferences for substrate colonization might be present, for some of the taxa there may
not have been enough organic matter and nutrients available to enhance colony formation
differences.

Bacterial populations from the \textit{Proteobacteria} groups were shown to respond
differently during the early days of biofilm formation when introduced to a different
substrate size. In fact, since the groups considered controls responded by either increased
abundance or maintaining their populations over those introduced to a different size
substrate, there is perhaps an advantage conferred by the substrate itself, and perhaps that
coupled with the amount of nitrogen in the microcosms could be the underlying factors in
the data seen.
References


Figure 1. A. Nitrate-Nitrite Nitrogen data collected on day 0, 1, 2 and 6 of the experiment when bacteria samples were collected. Values represent means (N=3). B Orthophosphate data collected on day 0, 1, 2 and 6 of the experiment when bacteria samples were collected. Values represent means (N=3).
Figure 2. Comparison of bacterial abundance on beads expressed per cm$^2$ of artificial substrate, based on DAPI (A) and FISH using Domain Bacteria probe (B). Values represent means (N=3) and standard errors shown on log scale. The x-axis labels represent the following: the numbers 0.1 or 5.0 to the left of the symbol (>) represent the bead diameter the bacteria were incubated on in situ; the numbers 0.1 or 5.0 to the right of the symbol (>) represent the bead diameter on which bacteria were inoculated for the microcosm experiment.
Figure 3. Comparison of bacterial abundance on beads expressed per cm$^2$ of artificial substrate based on FISH using taxon-specific probes: A. $\alpha$-Proteobacteria B. $\gamma$-Proteobacteria C. Cytophaga-Flavobacterium. Values represent means (N=3) and standard errors shown on log scale. The x-axis labels represent the following: the numbers 0.1 or 5.0 to the left of the symbol (>) represent the bead diameter the bacteria were incubated on in situ; the numbers 0.1 or 5.0 to the right of the symbol (>) represent the bead diameter on which bacteria were inoculated for the microcosm experiment.
A

B

C

0.1>0.1  5.0>0.1  5.0>5.0  0.1>5.0

# Bacteria per cm^2

1.00E+07

1.00E+05

1.00E+03

1.00E+01

Treatment
Figure 4. Comparison of bacterial abundance on beads expressed per cm² of artificial substrate based on FISH using species-specific probes: A. B. cepacia B. A. calcoaceticus. Values represent means (N=3) and standard errors shown on log scale. The x-axis labels represent the following: the numbers 0.1 or 5.0 to the left of the symbol (>) represent the bead diameter the bacteria were incubated on in situ; the numbers 0.1 or 5.0 to the right of the symbol (>) represent the bead diameter on which bacteria were inoculated for the microcosm experiment.
CHAPTER VI

General Discussion

The overall purpose of this work was to investigate the effects of substrate size, environmental features and temporal changes on bacterial communities in a Northeast Ohio stream. There were significant differences in abundance both temporally and spatially; in fact, there were many differences with certain features experimentally examined.

Generalized findings from all chapters include the following:

1. Total abundance expressed using DAPI staining is less likely to reveal a spatiotemporal response than using FISH that targets cellular rRNA in bacteria.
2. When temporal effects are incorporated, these effects seem to supersede other factors for most taxa examined.
3. _B. cepacia_ is a species of interest for a variety of reasons; specifically, though it is naturally occurring in streams, it can be implicated as a pathogen in both plants and animals. Abundance of this organism was generally less responsive to seasonal changes and experimental conditions.
4. Substrate size is shown to be a factor in bacterial abundance, whether examined as natural sediments or experimentally using artificial substrate. However, it is difficult to tease out the exact reasons why this is the case.
5. Organic matter, known to be so important in stream sediments was shown to be a less important factor in these investigations.

6. Location of artificial substrate in locations with varying velocity did not yield many differences.

7. Significant effects at the species level, e.g., *A. calcoaceticus*, are not necessarily reflected at the higher taxonomic level examined within the same group, in this case, *γ-Proteobacteria*.

8. The microcosm experiment showed that there were varying responses when bacteria are introduced to substrate of a different size.

Below each of these major points is expanded.

1. **DAPI versus FISH**

   Numbers of DAPI stained cells and those detected by FISH with the Domain *Bacteria* probe tended to follow different patterns, temporally, and also often spatially in the sediments. FISH targets rRNA, and therefore qualitatively reflect abundance of active cells at the time of sampling rather than total cells present (Poulsen et al. 1993).

   The high percentage difference between DAPI and FISH counts, particularly in the sediments, is an important consideration. Interestingly, the % difference was much
higher for both of the artificial substrata experiments than either of the field studies, for all habitats, particularly the sediment, although highest percentages varied among controls and amended substrate or placement (pool versus riffle). According to the Bouvier and del Giorgio survey (2003), where values are reported for 19 different freshwater systems, the % differences for the artificial substrate reported here were higher than their report of less than 40% (range from 1-100%). This under mines the use of artificial substrate as a means to quantify bacteria in sediments.

2. Temporal Effects

According to J. V. Ward (1989) time is the important fourth dimension in lotic ecosystems. It is difficult however to establish consistent temporal patterns even in one stream (e.g., Olapade and Leff 2004 compared to current data from Chapter I and II). Temporal effects incorporate the other variables as part of their suite of factors evoking responses from biota. The temporal scale for a bacterial population, although it interacts with higher taxa, is not necessarily the same scale as for other stream dwellers. Overall, pronounced seasonal changes occurred although the mechanisms behind these changes are unknown.
3. *B. cepacia*

*B. cepacia* was originally selected for examination of the species level response because prior studies suggest that it is likely to be consistently well represented under temporal investigation (Lemke et al. 1997). The ability of *B. cepacia* to use a variety of substrates may account for the lack of temporal changes in numbers (Palleroni 1975). *B. cepacia*’s very high genetic diversity with 9 currently known genomovars (Al-Bakri et al. 2004) is also potentially an important factor in how it responds in streams.

The documented ability of this species to be successful, regardless of environmental factors (e.g., temporal changes, organic matter, or even habitat in the stream) reveals its resilience and stability. These factors justify its study both ecologically and in other arenas of investigation.

4. **Substrate Size**

I expected to see suites of taxa that varied among the sediment size categories. This was not the case for the most part as responses of different taxa were generally similar. Differences among the three size categories of sediment did not yield the expected results and the hypothesis that there would be pronounced differences in community structure among the sediment size classes was not supported. The overall patterns of difference among size fractions for each taxon examined were similar in nearly all cases suggesting that the communities on the different fractions were highly
similar. Although it is difficult to elucidate the array of associated factors that contribute to the large differences among substrates of different sizes, the community responds as a whole in a similar fashion on the various sizes but their activity is obviously different, particularly on the smallest size substrate versus the largest sizes.

5. Organic matter

The amount of dissolved organic matter (DOM) in lotic systems is well documented (Wetzel 1984), as is the importance of bacterial uptake of DOM (Leff and Meyer, 1991, Amon and Benner 1996, Findlay and Sinsabaugh 1999). The leaves in the packets of the artificial substrate experiment were leaching out organic matter, in fact, the leaching was monitored in the lab during the period of incubation (data not shown), but it is possible this material was recalcitrant enough to not be taken up by the bacteria. Also, perhaps the fungal community was not sufficiently established in leaves in the packets, during the period of incubation, resulting in less suitable OM available to the colonizing bacteria (Ostrofsky 1997).

6. Location of packets in the stream channel

The lack of differences between natural sediments in a pool or riffle, or artificial substrata placed in a pool or riffle is somewhat surprising. The reach of the West Branch of the Mahoning where all studies were conducted, has changed in geomorphology
significantly over the last four years. The original pool, sampled in 2000-2001, was much deeper than the pool used for the experiment in 2003, but responses were similar. The overall lack of correlation with any of the abiotic features measured, other than a noticeable response to spring run off or post flooding responses also reflect the amazing ability of bacteria to survive a variety of habitat disturbances (Morita 1997).

7. Species level versus higher taxa

The example of the *A. calcoaceticus* response is yet another issue of scale level processes. This one species tended to respond differently than overall responses of the larger taxon (*γ-Proteobacteria*). According to Bergey's (2001), the phylum *Proteobacteria* are quite diverse (with over 1300 named species and more than 600 genera) and it is possible that under further investigation responses might be more variable than what the current taxonomic categories reveal. For example, the number of different species in each group, as well as variation in response, among species *r* are unknown.

8. Microcosm

Responses of bacteria incubated for the microcosm experiment to changes in substrate size were examined. After a month long incubation on one bead size (0.1 mm or 5.0 mm diameter glass beads) they were inoculated onto substrate of the opposite size or the same size in the laboratory. To my knowledge this has not been done before and if
there were inherent biological responses to an abiotic factor, it was quite possible a 
response would be seen. The bacteria did show some response, and where the more 
significant responses occurred was on substrate of the same size. There seemed to be 
some feature of colonizing, or surviving that worked better on substrate of the same size 
as the cells were originally grown on.

General Conclusion

Temporal responses were quite obvious overall but the cause behind the patterns 
was generally elusive. Substrate size is clearly important in determining bacterial 
number and more than surface area determines how effectively bacteria can colonize and 
grow. Generally, different taxa exhibited similar responses to differences in substrate 
grain size, indicating that some similar physical, biological or chemical factor influences 
each group in a similar manner.
References


