CAUSES AND FUNCTIONAL CONSEQUENCES OF DENITRIFYING BACTERIA COMMUNITY STRUCTURE IN STREAMS AFFECTED TO VARYING DEGREES BY WATERSHED URBANIZATION

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Si-Yi Wang

Department of Biology
Duke University

Date:_____

Approved:

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Justin P. Wright, Supervisor

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Emily S. Bernhardt

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Claudia K. Gunsch

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Dean L. Urban

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Rytas J. Vilgalys

Dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the Department of Biology
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ABSTRACT

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Abstract

Human welfare depends heavily on ecosystem services like water purification and nutrient cycling. Many of these ecosystem services, in turn, rely on reactions performed by microbes and yet remarkably little is known about how anthropogenic impacts are affecting the structure and function of microbial communities. To help address this knowledge gap, this dissertation uses field surveys and laboratory experiments to examine how watershed urbanization affects microbial communities in receiving streams. We focus on a specific functional group and its associated function – the denitrifying bacteria and denitrification. Denitrifying bacteria use reactive nitrogen and organic carbon as substrates to perform denitrification. Denitrification is one of the few ways to permanently remove reactive nitrogen from ecosystems. Since excess reactive nitrogen in water contributes to serious water quality and human health problems like toxic algal blooms and bowel cancer, denitrification in streams can be considered a valuable ecosystem service. Watershed urbanization, however, may alter the structure of denitrifying bacteria communities in ways that constrain their capacity to remove reactive nitrogen from streams.

Watershed urbanization leads to drastic changes in receiving streams, with urban streams receiving a high frequency of scouring flows, together with increased nutrient (nitrogen and carbon), contaminant (e.g., heavy metals), and thermal pollution. These changes are known to cause significant losses of sensitive insect and fish species from urban streams. Microbes like denitrifying bacteria may be similarly affected. In the first part of this dissertation, we describe results from four repeated surveys of eight central North Carolina streams affected to varying degrees by watershed urbanization. For each stream and sampling date, we characterized both overall and denitrifying bacterial

communities and measured denitrification potentials. Differences in overall and denitrifying bacteria community composition were strongly associated with the urbanization gradient. Denitrification potentials, which varied widely, were not significantly associated with substrate supply. By incorporating information on the community composition of denitrifying bacteria together with substrate supply in a linear mixed-effects model, we explained 45% of the variation in denitrification potential ($p < 0.001$). Results suggest that 1) watershed urbanization can lead to significant changes in the composition of bacterial communities in streams and 2) such changes may have important functional consequences.

The second part of this dissertation examines how urbanization-driven changes to the structure of denitrifying bacteria communities might affect the way they respond to stress or disturbance. Some communities can resist changes to functionality in response to disturbance, potentially as a result of previous exposure and subsequent adaptation (legacy hypothesis) or high diversity (insurance hypothesis). We compare the resistance of two structurally distinct denitrifying bacteria communities to experimental disturbances in laboratory microcosms. Communities originated from either a polluted, warm urban streams or a relatively pristine, cool forest stream. In this case, the two communities had comparable compositions, but forest communities were more diverse than their urban counterparts. Urban communities experienced significant reductions in denitrification rates in response to the most severe increased pollution and temperature treatments, while forest communities were unaffected by those same treatments. These findings support the insurance, but not the legacy hypothesis and suggest that the functioning of urban streams may be more susceptible to further environmental degradation than forest streams not heavily impacted by human activities.

In the third part of this dissertation, we discuss results from a one-time survey of denitrifying bacteria communities and denitrification potentials in 49 central North Carolina streams affected to varying degrees by watershed urbanization. We use multivariate statistics and structural equation modeling to address two key questions: 1) How do different urban impacts affect the structure of denitrifying bacteria communities and 2) How do abiotic (e.g., temperature) versus biotic (denitrifying bacteria community structure) factors affect denitrification potentials in urban streams? Denitrifying bacteria community structure was strongly affected by the urban impacts measured. Community composition responded to increased temperatures, substrate supply, and contamination, while diversity responded negatively to increased temperatures and hydrologic disturbance. Moreover, increased temperatures and substrate supply had significant positive effects, while urbanization-driven changes to denitrifying bacteria community structure had significant *negative* effects on denitrification potential. The structural equation model captured 63% of the variation in denitrification potential among sites and highlighted the important role that microbial community structure can play in regulating ecosystem functioning. These findings provide a novel explanation for recent observations of decreasing denitrification efficiency with increasing urbanization. Ultimately, we hope findings from this dissertation will help inform more effective stream management and restoration plans and motivate ecologists to consider including microbial community structure in ecosystem models of microbe-mediated processes.

Contents

List of Tables

List of Figures

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1. Introduction

1.1 Microbes and ecosystem functioning

One of ecology's most urgent challenges is to understand how human driven changes in species assemblages may alter ecosystem functioning (Hooper et al. 2005; Naeem and Wright 2003). Microbes, including bacteria, fungi, and archaea, mediate many of the key biogeochemical transformations that underpin ecosystem functioning (Zak, Blackwood, and Waldrop 2006). For instance, microbes decompose organic matter, purify water, and sustain soil fertility – biogeochemical processes critical to human health and welfare. Microbes are also the most ubiquitous, abundant, and diverse group of organisms on Earth (Pace 1997). In fact, bacteria and archaea alone contain 85-130 Pg of nitrogen, which represents the largest pool of nitrogen in living organisms and is ten times the amount found in plants (Whitman, Coleman, and Wiebe 1998). Clearly, our understanding of how humans are affecting ecosystem processes would be incomplete without an explicit consideration of the microbes that dominate them in so many ways (Allison and Martiny 2008).

Ecologists generally agree that shifts in plant community structure can alter ecosystem functioning (reviewed by Loreau et al. 2001). The same could also be true for microbial communities and yet most major ecosystem models, such as the CENTURY model of plant-soil nutrient cycling (Parton et al. 1987), completely ignore microbes, despite having many parameters related to microbe-mediated processes. Microbial community structure has been omitted from ecosystem models partly because of the methodological difficulties associated with cataloging the exceedingly complex microbial communities in the environment (Zak, Blackwood, and Waldrop 2006). Furthermore, the supposedly unlimited dispersal, rapid growth, and high abundance and diversity of microbial communities have led to the widely-held assumption that

neither the composition nor diversity of microbial communities can limit ecosystem process rates.

Fortunately, molecular technologies have now advanced spectacularly (Zak, Blackwood, and Waldrop 2006), allowing detailed descriptions of the "unseen majority" that drive Earth's biogeochemical cycles (Falkowski, Fenchel, and Delong 2008; Whitman, Coleman, and Wiebe 1998). These advances have given us the tools to question the assumption that microbial community structure does not influence ecosystem functioning. Indeed, a growing body of evidence now suggests that microbial community structure can play a critical role in regulating ecosystem process rates (Cavigelli and Robertson 2000; Philippot et al. 2011; Schimel 1995; Wittebolle et al. 2009). To contribute to the now rapidly expanding field of microbial ecology (Prosser et al. 2007), this dissertation applies theories from ecological research on 'macrobes' (e.g., plants and animals) to a study of the causes and functional consequences of microbial community structure in streams affected to varying degrees by watershed urbanization.

1.2 Urbanization and streams

Of the major human activities affecting ecosystems, land use change is expected to have the "largest global impact on biodiversity by the year 2100" (Sala et al. 2000). Urbanization, the conversion of land to residential and industrial uses, is an especially prominent type of land use change, both in terms of its ecological impact and the pace of alterations (McKinney 2002). Incredibly, the world's urban population is expected to double by 2050, making it equal to the world's *total* population in 2004 (UNPD 2008). The ecological impacts of urbanization on water quality, local climates, and energy and nutrient flows are profound (Grimm et al. 2000; Redman and Jones 2005; Vitousek et al. 1997). The environmental gradients generated by varying degrees of urbanization intensity therefore provide an "unexploited opportunity" to answer general ecological

questions (McDonnell and Pickett 1990). Urban ecosystems are thus not only immensely relevant in a rapidly urbanizing world, but also opportune places to study the functional consequences of microbial responses to anthropogenic disturbance.

As low-lying points in landscapes, streams are strongly affected by the many chemical and hydrologic consequences of watershed urbanization (reviewed by Walsh et al. 2005). For instance, urban streams generally have higher temperatures, higher nutrient (carbon, nitrogen, and phosphorus) concentrations, increased contamination by toxic chemicals, and increased severity and frequency of hydrologic disturbances relative to reference streams (Paul and Meyer 2001; Walsh, Fletcher, and Ladson 2005). A large body of literature has documented substantial shifts in the structure of stream algal, insect, and fish communities in response to urban impacts (reviewed by Roy et al. 2009). Based on a very limited number of studies to date, the composition of microbial communities in stream biofilms (Lear and Lewis 2009) and sediments (Perryman, Rees, and Walsh 2008) may be similarly sensitive to urban impacts. Streams are thus suitable places to study the effects of urbanization on microbial communities.

In addition to being useful model systems for studying urbanization, streams are also important and valuable ecosystems in their own right. Streams are conduits of fresh water for growing crops and livestock, habitats for many endangered and threatened species, and home to commercially valuable fish and shellfish stocks. Moreover, humans use streams to sustain drinking water supplies and as important recreational areas (UNEP 2005). Like riparian wetlands, streams are also biogeochemical hotspots for the microbial removal of nutrients like nitrogen that can severely degrade water quality when present in excess amounts (Bernhardt et al. 2003; Peterson et al. 2001; UNEP 2005).

Recent studies, however, have found that the efficiency with which streams remove nutrients may be constrained by the impacts of urbanization (Grimm et al. 2005;

Inwood, Tank, and Bernot 2007; Meyer, Paul, and Taulbee 2005; Mulholland et al. 2008), possibly through changes in microbial community structure. These findings have prompted scientists studying urban streams to identify an improved understanding of microbial communities, particularly those involved in nitrogen cycling, as one of the most important research frontiers in urban stream ecology (Bernhardt et al. 2008; Wenger et al. 2009). This dissertation addresses this challenge by examining the denitrifying bacteria (denitrifiers) and denitrification. Denitrification is a key part of the nitrogen cycle and is one of the few ways to remove excess nitrogen from water bodies.

1.3 Humans and nitrogen

Before moving on to a more thorough discussion of denitrifiers and denitrification in urban streams, some background information on why excess nitrogen is such an important environmental problem is needed.

In the early twentieth century, the rapid growth of human populations fueled rising demand for food, conditions that motivated the invention of the Haber-Bosch process of producing reactive nitrogen (N_r) to make fertilizers. Since that time humans have benefitted greatly from resulting increases in agricultural yield, but the associated costs of excess N_r in the environment are substantial as well (Sutton et al. 2011). A significant fraction of the N_r applied to landscapes is not taken up by crops, but is instead lost as runoff that pollutes streams, rivers, lakes, and coastal zones. In fact, human activities, including fertilizer use, industry (e.g., nylon production), planting of N-fixing crops, and fossil fuel combustion, have led to a doubling of the Earth's annual supply of N_r over the last century (Galloway et al. 2004; Vitousek et al. 1997). Much of this excess N_r makes its way into surface waters (Turner and Rabalais 2003), leading to serious water quality and human health problems (Townsend et al. 2003; Vitousek et al. 1997).

N_r, otherwise known as biologically available nitrogen, is a key limiting nutrient for many organisms and contrasts with the unreactive and biologically unavailable nitrogen (N_2) that makes up most of the Earth's atmosphere. Excess N_r in water bodies contributes to eutrophication, toxic algal blooms, and regional hypoxia in coastal zones (Sutton et al. 2011; Vitousek et al. 1997). Excess N_r in drinking water have also been linked to blue baby syndrome (methemoglobin) and certain cancers (Townsend et al. 2003). Moreover, nitrogen pollution can strongly affect aquatic communities, leading to local species extinctions and loss of biodiversity, with potentially negative consequences for ecosystem functioning and services (Chapin et al. 2000; UNEP 2005; Vitousek et al. 1997). Nitrogen pollution is now widely recognized as a serious and costly environmental problem that shows no signs of abating in the near future (UNEP 2005).

There are many ways of mitigating nitrogen pollution in aquatic ecosystems, the most important of which is preventing N_r from entering water in the first place. For urban streams in particular, major sources of N_r are thought to include food, drinking water, and industrial chemicals (Bernhardt et al. 2008). The N_r in food, water, and industrial chemicals generally enters sewer networks that may or may not include wastewater treatment plants. Resulting effluents are often discharged directly into streams, along with any of the remaining N_r. To help reduce N_r inputs into urban streams, we must improve wastewater treatment removal of N_r, increase access to wastewater treatment, and repair sewer leaks (Bernhardt et al. 2008; Sutton et al. 2011). Nitrogen pollution, however, is a complex problem that requires a multi-pronged approach (Galloway et al. 2008). So, while reducing Nr inputs is of the utmost importance, other strategies, including encouraging the removal of Nr in riparian wetlands and streams through denitrification, are also needed to effectively address nitrogen pollution (Bernhardt et al. 2008).

1.4 Denitrifying bacteria (denitrifiers) and denitrification

Denitrification is essentially the stepwise reduction of nitrate, the main form of Nr entering streams, to unreactive N_2 that can then escape to the atmosphere, effectively reversing the Haber-Bosch process (Groffman et al. 1999; Payne 1981; Zumft 1997). Denitrification is thought to be largely mediated by bacteria and only occurs under hypoxic or anoxic conditions and requires Nr and organic carbon as substrates (Zumft 1997). Much of the nitrate applied to landscapes can be removed by denitrification in riparian wetlands and streams before reaching coastal zones (Howarth et al. 1996; Peterson et al. 2001). Stream algae and microbes can also take up nitrate through assimilation, but since this organic nitrogen can be remineralized later as organisms die and decompose, the process cannot be considered permanent. Denitrification, on the other hand, is one of the few ways to permanently remove nitrate and, therefore, presents an important mitigation strategy for nitrogen pollution.

It is important to recognize, however, that denitrification has both costs and benefits. The process does not always go to completion and can sometimes produce intermediate products (i.e., products other than N_2) that include nitrous oxide (N_2O), a potent greenhouse gas that exacerbates climate change and stratospheric ozone destruction. Factors thought to encourage the amount of N_2O produced relative to N_2 include high Nr availability, high oxygen concentrations (Firestone, Firestone, and Tiedje 1980), high sulfide concentrations (Sorensen, Tiedje, and Firestone 1980), and low pH (Koskinen and Keeney 1982). The ratio of N_2O to N_2 produced by denitrification may also be controlled by the relative abundances of denitrifiers with versus without the gene for N_2O reductase (Philippot et al. 2011). Recent research suggests that as much as one-third of denitrifiers capable of reducing nitrite cannot reduce N_2O (Jones et al. 2008).

There have been few studies of N_2O emissions from denitrification in streams, relative to those in soils, but a recent study suggests that most streams release N_2O into the atmosphere and urban streams in particular release the greatest amounts (Beaulieu et al. 2011). Given these findings, further research is needed to improve our understanding of the relationship between denitrifier community structure and N_2O emissions in urban streams. While this dissertation does not directly address this relationship (i.e., we did not measure N_2O emissions), we did examine patterns of distribution for nitrite reducing and N_2O reducing denitrifiers.

In addition to being ecologically significant, the denitrifiers that mediate denitrification also provide an ideal model for studying microbial responses to anthropogenic disturbance. The functional genes involved in making denitrification enzymes have been described, allowing the use of culture-independent, molecular methods to track changes in denitrifier community structure. Since most microbes in the environment cannot be easily isolated and cultured in the laboratory, the ability to use culture-independent methods is an important advantage. Moreover, denitrifiers are a phylogenetically diverse group (concentrated mostly along different subclasses of the *Proteobacteria*) with large variations in physiology and life history traits (Zumft 1997). That is, denitrifier species differ in the way they respond to the environment. For example, they do not all have the same optimal temperature for growth (Philippot and Hallin 2005). Denitrifier communities are therefore likely to change in response to the selective pressures imposed by urbanization.

Denitrifiers are also ideal for studying the functional consequences of microbial community shifts. Denitrifiers differ not only in how they respond to stress or disturbance, but also in how efficient they are at denitrification. For instance, different species have different reaction rates and affinities for nitrate (Philippot and Hallin 2005).

Denitrifier community structure can be an important control on denitrification rates (Philippot and Hallin 2005; Wallenstein et al. 2006). That is, even under the same abiotic conditions, denitrifier communities with different compositions and/or diversity can have very different denitrification rates (Cavigelli and Robertson 2000; Holtan-Hartwig, Dorsch, and Bakken 2000; Rich et al. 2003; Wittebolle et al. 2009). Since we can measure denitrification rates (Groffman et al. 2006), researchers can readily track changes in both denitrifier community structure *and* function in response to environmental change.

1.5 Denitrifiers and denitrification in urban streams

Most denitrifiers are facultative anaerobes (Zumft 1997), using oxygen as the electron acceptor for organic matter oxidation whenever available and nitrate as the next best alternative electron acceptor under hypoxic or anoxic conditions. Urban streams typically have low oxygen and high nitrate and organic carbon concentrations, as well as higher temperatures (Walsh et al. 2005). Recent findings of reduced denitrification efficiency (i.e., percent of nitrate removed by denitrification) in urban streams (Inwood, Tank, and Bernot 2007; Mulholland et al. 2008) are thus surprising from an abiotic standpoint. That is, if we only saw denitrifiers as abiotic catalysts, we would expect denitrification rates to increase in step with increases in substrate supply.

Denitrifiers, however, are not abiotic catalysts, but living organisms that respond to all the changes in an environment. Urbanization affects more than just oxygen and substrate concentrations; it also imposes many stressors on stream inhabitants, such as contamination and increased severity and frequency of hydrologic disturbances (Walsh et al. 2005). These stressors may drive changes in denitrifier community structure that could, in turn, constrain denitrification (Wallenstein et al. 2006). In the single published study on this topic to date, signficant differences in denitrifier community composition were found between an urban versus non-urban stream (Perryman, Rees, and Walsh

2008). This dissertation goes beyond that study by surveying more than two streams and by examining both denitrifier community structure *and* function.

We can think of two ways by which urbanization-driven changes to denitrifier community structure might lead to reduced denitrification efficiency. One mechanism focuses on potential links between denitrifier diversity (i.e., richness and evenness) and denitrification rates. The stressors found in urban streams might lead to loss of diversity, which could, in turn, decrease denitrification rates (see next section). The other mechanism focuses on potential links between denitrifier community composition and denitrification rates. Contamination by multiple toxic chemicals in urban streams might select for highly tolerant denitrifiers. For plants, tolerance mechanisms are typically costly and accompanied by tradeoff traits that would otherwise be unfavorable to organism fitness (Grime 1977). The same could also be true for microbes. Consistent with this theory, metal tolerant denitrifier communities had lower denitrification rates relative to metal sensitive communities (Holtan-Hartwig et al. 2002). Furthermore, multiple metal resistant bacteria in freshwater lakes expressed fewer enzymes than single metal resistant bacteria (De Souza et al. 2007). Thus, selection for highly tolerant denitrifiers in response to stress might have a negative impact on denitrification.

There are numerous other theories on how biotic community structure might be linked to ecosystem functioning, with perhaps the most controversial concepts coming from studies of plant diversity and ecosystem productivity (Hector et al. 1999; Hooper et al. 2005; Loreau et al. 2001; Yachi and Loreau 1999). We argue that some of these concepts may also be applicable to microbial communities and others less likely.

1.6 Biodiversity and ecosystem functioning: a microbial view

When investigating biodiversity and ecosystem functioning, ecologists generally focus on richness (i.e., the total number of species) rather than evenness (i.e., the relative

abundances of species) (Hillebrand, Bennett, and Cadotte 2008). Highly uneven communities are dominated by a few, highly abundant species while perfectly even communities have equal abundances for all member species. For naturally occurring (as opposed to artificially assembled) microbial communities, richness may not be as informative as evenness for two key reasons: 1) it is nearly impossible to catalog all of the microbial species present in a sample, so our estimates of microbial richness are almost always underestimates and 2) it is difficult to imagine that any ordinary stress or disturbance aside from a catastrophic event (e.g., large meteorite impact) could lead to significant and sustained local extinctions of microbial taxa (Balser, Kinzig, and Firestone 2002). For these reasons, microbial ecologists tend to focus on evenness or composition rather than richness (Balser, Kinzig, and Firestone 2002) as potential drivers of ecosystem functioning.

Broadly speaking, ecologists have identified two types of mechanisms for a positive relationship between biodiversity and ecosystem functioning: complementarity or facilitation and the sampling effect. Complementarity or facilitation arises when a diverse community is able to access more of a limiting resource (e.g., sunlight or water) and, therefore, support higher rates of ecosystem functioning than a less diverse community (Hooper 1998). Greater access to resources may occur because of niche partitioning between community members with different traits (complementarity) or because of a species' modification of the environment in a way that benefits other community members (facilitation). However, the identities of species within the community (i.e., composition) may be just as important as diversity in promoting access to resources; this idea is known as the sampling effect (Fridley 2001). In other words, the addition of just a few individuals of the 'right' species can be enough to partition

resources or modify the environment in a way that sustains high rates of ecosystem functioning (Schwartz et al. 2000).

The sampling effect reflects the importance of species identities in controlling ecosystem functioning. The basic concept is that diverse communities have a greater likelihood of including species that 1) are well suited to the environmental conditions at the site, 2) are especially good at that particular function (e.g., high maximum size or growth rate for biomass production), 3) have traits that complement those of other species in the community, and/or 4) can modify the environment in a way that facilitates other species in the community (Fridley 2001). The sampling effect can affect not only the absolute rate of ecosystem functioning, but also the stability of ecosystem functioning through fluctuating environmental conditions (i.e., across space and/or time), a concept known as the insurance hypothesis (Yachi and Loreau 1999). The insurance hypothesis predicts greater functional resistance to stress or disturbance for communities with high versus low diversity.

We contend that the sampling effect may be more applicable to naturally occurring microbial communities than complementarity or facilitation. Given the high levels of microbial diversity in most environments, random additions of species are theoretically unlikely to increase ecosystem functioning except through the sampling effect, particularly if the conditions that led to low rates of ecosystem functioning in the first place remain unchanged. This does not mean that ecologists studying biodiversity and ecosystem functioning should shy away from using microbial communities as experimental model systems. What it does mean, though, is that denitrifier community composition may be more important than diversity per se in determining the absolute value and stability of denitrification rates in fluctuating environments.

Given the potential links between denitrifier community structure and function, stream restoration and management plans may need to consider not only plant and animal, but also microbial communities as critical components of a functionally intact stream. That is, to effectively minimize the export of excess Nr to downstream ecosystems, we must gain a mechanistic understanding of potential microbial constraints to denitrification. Findings from this dissertation may demonstrate the vulnerability of seemingly infallible microbial communities to environmental degradation and point to new management or restoration priorities for reducing specific urbanization impacts (e.g., contamination by heavy metals) that may be preventing the assembly of highly efficient denitrifier communities. Moreover, we believe that the methodological and statistical approaches used in this dissertation may be widely adapted for studying other microbial communities and ecosystem processes.

1.7 Dissertation Outline

This dissertation consists of three chapters examining the effects of watershed urbanization on the structure and function of denitrifier communities in streams. Chapter two, which is titled "Watershed urbanization alters the composition and function of bacterial communities in streams", is based on repeated surveys of denitrifier communities along an urbanization gradient made up of eight streams in the Raleigh-Durham metropolitan area of North Carolina (USA). In addition to characterizing the structure of denitrifier communities, we also measured potential denitrification rates (i.e., the maximum rate achievable by the extant community). We use linear mixed-effects models to determine whether including parameters representing denitrifier community composition improves our ability to capture variations in denitrification potential among sites.

Chapter three, which is titled "Bacterial diversity is linked to higher functional resistance", is based on results from laboratory microcosm experiments set up to test how structurally distinct communities differed in their ability to resist changes to function in response to disturbance. Using sediments from the end point streams from the urbanization gradient described in chapter one, we set up microcosms with either urban or forest stream communities and then subjected them to disturbance treatments before measuring denitrification rates. We explore whether differences in composition and/or diversity help explain differences in functional resistance.

Chapter four is titled "Causes and functional consequences of denitrifying bacteria community structure in streams affected to varying degrees by watershed urbanization." Like chapter one, this chapter is based on an observational survey of stream denitrifier communities and denitrification potential along a gradient of watershed urbanization in the Triangle region of the North Carolina Piedmont (USA). Chapter four moves beyond chapter one, however, by including a larger number of streams (n = 49) and by using structural equation modeling to evaluate multiple hypothesized pathways driving observed differences in denitrifier community structure and function among sites. Chapter five concludes the dissertation by summarizing findings from all three papers and providing suggestions for future research.

2. Watershed urbanization alters the composition and function of bacterial communities in streams

2.1 Introduction

Streams occupy low lying points in landscapes and are thus strongly affected by the detrimental impacts of watershed urbanization (reviewed by Walsh et al. 2005). An extensive body of research has documented significant losses of sensitive insect and fish species from streams in response to urbanization (reviewed by Roy et al. 2009). In contrast, relatively little is known about how urbanization affects bacterial community composition in streams (Wenger et al. 2009). Given that bacteria mediate many of the biogeochemical transformations underpinning ecosystem functioning (Falkowski, Fenchel, and Delong 2008), changes to the composition of stream bacterial communities in response to urbanization have the potential to alter ecosystem functioning in streams (Allison and Martiny 2008).

There are reasons to expect watershed urbanization to alter bacterial community composition in streams – urban streams are highly stressful environments that receive severe and frequent physical disturbance through scouring flows, together with increased streamwater temperatures and contaminant (e.g., heavy metals) and nutrient inputs (Meyer, Paul, and Taulbee 2005; Walsh et al. 2005). Physical disturbance, as well as thermal and chemical changes, have been found to strongly regulate bacterial community composition in streams (reviewed by Findlay 2010) and terrestrial systems (Allison and Martiny 2008; Waldrop and Firestone 2006; Wang, Hou, and Guo 2010). We therefore expected watershed urbanization to cause substantial changes to the composition of stream bacterial communities. In the single published study on this topic to date, Lear *et al*. found that bacterial community composition in stream biofilms was

significantly different between streams in urban versus rural watersheds (Lear and Lewis 2009).

It is less clear how urbanization might affect the composition and function of bacterial functional groups (i.e., groups whose members all share the ability to perform a particular function). One particularly important functional group and function in streams is denitrifying bacteria (denitrifiers) and denitrification. Denitrification is the transformation of nitrate to nitrogen gas and is one of the few ways to permanently remove nitrate from surface waters. The global supply of nitrate has more than doubled over the last century (Galloway et al. 2004), particularly in urban streams (Bernhardt et al. 2008; Walsh et al. 2005), leading to serious water quality and human health problems (Sutton et al. 2011; Townsend et al. 2003; Vitousek et al. 1997). Denitrification by stream denitrifiers can play a key role in mitigating nitrogen pollution by preventing nitrate from entering downstream ecosystems (Bernhardt et al. 2008; Mulholland et al. 2008; Payne 1981). Our goal in the study was to examine whether watershed urbanization affects the composition of overall bacterial communities and the composition and function of denitrifier communities in streams.

Most denitrifiers are facultative anaerobes, using oxygen as the electron acceptor for organic matter oxidation whenever available and nitrate as the next best alternative electron acceptor under hypoxic or anoxic conditions. Denitrification is thus expected to be highest in habitats with low dissolved oxygen and high nitrate and organic carbon substrate supply. Urban streams tend to have low oxygen and high substrate concentrations (Walsh et al. 2005), making them seemingly ideal places for denitrification. Urbanization, however, affects more than just oxygen and substrate concentrations; it also imposes many stressors on stream inhabitants, such as contamination, high temperatures, and hydrologic disturbances (Walsh et al. 2005).

These stressors may drive changes in denitrifier community composition (Perryman, Rees, and Walsh 2008) that could, in turn, lead to altered denitrification rates (Wallenstein et al. 2006).

We propose two mechanisms by which watershed urbanization could affect stream denitrification. The first mechanism focuses on direct effects of urbanization on denitrification through changes to nitrate and organic carbon concentrations (pathway A in Figure 1). The second mechanism focuses on indirect effects of urbanization on denitrification through changes to denitrifier community composition (pathway B in Figure 1). To compare the relative importance of the two mechanisms, we examined both denitrifier community composition and substrate concentrations as potential controls of denitrification potential in streams affected to varying degrees by watershed urbanization.

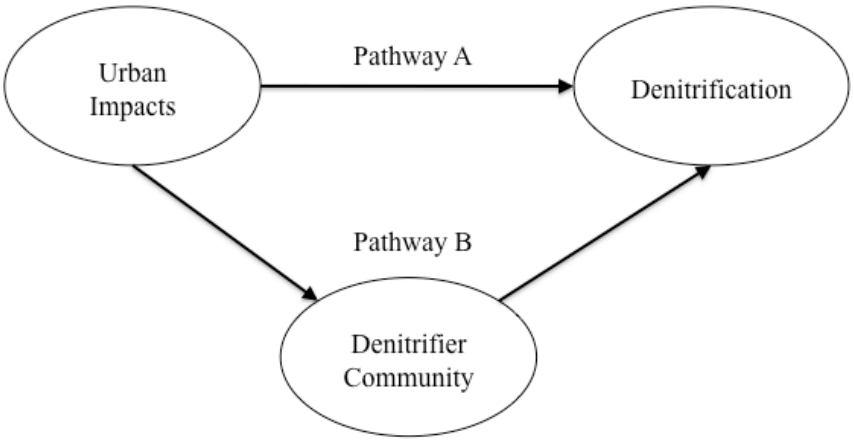

Figure 1: Pathways through which watershed urbanization might alter denitrification rates in streams

2.2 Methods

2.2.1 Study streams

We surveyed microbial communities and measured denitrification potentials in eight study streams located in the Raleigh-Durham area in the Piedmont region of North

Carolina (USA) (Table 1). We selected the study streams to represent a gradient of watershed urbanization, with percent impervious cover (% IC) ranging from 1 to 39%. Watershed land cover metrics were calculated based on the 2001 National Land Cover Dataset and the associated Impervious Surface Cover dataset, both from the United States Geological Survey Seamless Server. Within each study stream, we established a permanent study reach from which to collect samples during the course of the research effort (June 2008 to July 2009). Further details on the physical conditions (e.g., total degree-days, hydrologic flashiness, etc.) and the macroinvertebrate communities at each site can be found in (Sudduth et al. 2011; Violin et al. 2011).

2.2.2 Sediment collection

We collected sediments from streams on four sediment sampling dates – sampling date 1 (June 2008), 2 (December 2008), 3 (June 2009), and 4 (July 2009). Mud Creek and Lower Mud were dry on sampling date 4, so sediments were not collected from those streams on that date. To collect sediments, we randomly selected five points along each ~ 100 m study reach. We then demarcated a 2 m segment of streambed (i.e., entire wetted width) upstream and downstream of each selected point and used PVC corers (6.35 cm diameter) to take multiple sediment cores in each sampling area until a total volume of at least 4,024 cm^3 was collected. Sediment cores were sieved (2 mm opening) and composited in the field, resulting in a single composited sample from each site on each date. Sediment subsamples for molecular analyses and denitrification potentials were kept on ice for transport to the laboratory and then stored at -80˚C and 4˚C, respectively. Denitrification potentials were measured within 48 hours of sample collection.

Table 1: Study streams, ranked in order of watershed percent impervious cover

Stream	Watershed		NO_3-N [1]	TOC [1]	Total metals [2]	Total degree days[3]	Flashi-ness[4]	EPT rich-ness[5]
	Impervious cover (%)	Develop-ment (%)						
Mud Creek	0.5	4.4	0.11	4.78	16	11018	0.04	12.0
Stony	3.4	24.4	0.20	4.17	29	10691	0.01	9.0
Lower Mud	9.5	58.6	0.15	5.29	20	11418	0.01	2.5
Pott's	9.9	27.4	0.08	4.68	29	11020	0.04	8.5
Upper Mud	11.0	66.9	0.13	6.29	34	11450	0.26	0.0
Cemetery	19.1	98.0	1.44	2.15	30	11470	0.14	2.0
Ellerbee	20.8	88.7	0.22	7.62	24	12167	0.09	3.5
Goose	39.4	100.0	0.20	15.17	34	12899	0.17	0.0

Notes:
[1] Mean streamwater concentrations (mg L^{-1}) taken between June 2008 and July 2009
[2] Measure of cumulative heavy metal loading in July 2009 sediment samples
[3] Total degree-days calculated with daily minimum and maximum temperatures using the double triangle method (Roltsch et al. 1999) and data taken between May 2007 and June 2007 (Sudduth et al. 2011).
[4] Flashiness estimated from changes in hourly discharge between May and June 2007 (Sudduth et al. 2011).
[5] Mean number of macroinvertebrate species belonging to *Ephemeroptera*, *Plecoptera*, and *Trichoptera* found in 2006 and 2007 surveys (Violin et al. 2011). EPT richness is often used as an indicator of water quality; EPT species tend to occur in clean, well oxygenated waters (Rosenberg and Resh 1993).

2.2.3 Water chemistry

We collected streamwater samples from each site on the same day of sediment sampling and at least once per month from June 2008 to July 2009. Samples were field filtered through Whatman GF/F filters (Whatman, Piscataway, NJ, USA) and kept on ice for transport to the laboratory. All samples were stored at 4°C and analyzed for nitrate and total organic carbon (TOC). We measured nitrate with an ion chromatograph

equipped with an AS18 anion column and KOH eluent generator (Dionex, Sunnyvale, CA, USA). We measured TOC as non-purgeable organic carbon with a TOC analyzer (Shidmadzu Corporation, Kyoto, Japan). For all statistical analyses of nitrate and TOC concentrations, we used all available measurements from the month of each sediment sampling date to calculate a mean value for that particular site and sediment sampling date.

2.2.4 Sediment heavy metals

While pharmaceuticals, herbicides, and other toxic chemicals can also contaminate urban streams, we chose to focus primarily on heavy metals as an indicator of overall contamination intensity, because previous work has documented denitrifier community shifts in response to heavy metal contamination (Throback et al. 2007) and heavy metal concentrations are relatively easy to measure. We measured the concentration of nine heavy metals, including silver (Ag), aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn), in sediments collected in June 2009.

To measure heavy metal concentrations, sediments were re-sieved (1mm opening), dried at 60°C for 48 hours, and then weighed out into three replicate 1 g subsamples per site per date for digestion (EPA method 3050B). Digestion involved adding 10 mL of 50% nitric acid, heated at 95°C, followed by another 5 mL of nitric acid (also heated to 95°C) to each sample. 10 mL of hydrogen peroxide were then added before filtering (Whatman #41). Digested samples were analyzed for trace metals by inductively coupled plasma-mass spectrometry (Perkin-Elmer Elan 6000 ICP-MS, Perkin-Elmer, Waltham, MA, USA). For every 35 samples analyzed, we also processed three replicates of certified reference materials STSD-3 (NRC, Ottawa, Canada) and two method blanks.

To correct for differences in organic carbon content among sediment samples, we standardized all heavy metal concentrations using ash-free dry mass (AFDM). AFDM is the amount of dry mass of a sediment sample that is organic and can, therefore, be combusted. To determine AFDM values, we weighed out three replicate 5 g subsamples per site per date, dried at 60°C for 48 hours, weighed to get dry mass, combusted at 400°C for 4 hours, and weighed again to get combusted mass. The difference between dry and combusted mass, divided by dry mass is AFDM. Standardization is necessary for comparing concentrations of substances, like heavy metals and organic pollutants, in sediments that vary in their physio-chemical properties. While there are many normalizers available, organic carbon content is often used for standardizing heavy metal concentrations (Liu et al. 2003; Loring 1991).

In addition to the concentrations of individual heavy metals, we also calculated an additional metric (total metals) to represent the cumulative heavy metal load in sediments by categorizing each heavy metal concentration into a quantile category (i.e., 1 to 5) and then summing quantile values across all nine measured heavy metals for each site. Quantile values give equal weight to all metals, as opposed to a simple sum of concentrations, which would weigh metals with the highest concentrations (that may not be the most toxic) most heavily.

2.2.5 Sediment bacterial community composition

The focus of this study was on assessing variability in community composition among sites, rather than within each site. We therefore extracted DNA from sediment subsamples taken from field composited (per site and sampling date) sediment cores. Extractions were done using PowerSoil kits (MoBio Laboratories, Carlsbad, CA, USA), according to manufacturer instructions. Samples collected on sampling dates 1 and 2 were extracted in triplicate, while those collected on sampling dates 3 and 4 were

extracted in duplicate. We used extractions from sampling dates 3 and 4 to characterize overall bacterial communities and extractions from sampling dates 1, 2, 3, and 4 to characterize denitrifier communities.

We amplified bacterial 16S rRNA genes with the universal bacterial primer set 8F (5'-AGAGTTTGATCCTGGCTCAG, HEX labeled) (Liu et al. 1997) and 1389R (5'-ACGGGCGGTGTGTACAAG) (Osborn, Moore, and Timmis 2000) using Apex 2x Taq Master Mix (Genesee Scientific, San Diego, CA, USA). Each of 28 polymerase chain reaction (PCR) cycles consisted of 45 seconds at 94°C, 45 seconds at 58°C, and 90 seconds at 72°C. Three separate 16S rRNA PCRs were done for each extraction (i.e., two extractions per site on each date).

We amplified denitrifier DNA with two functional gene primer sets – *nirK* and *nosZ*. The *nirK* primer set was nirK1F (5'- GG(A/C)ATGGT(G/T)CC(C/G)TGGCA, FAM labeled) and nirK5R (5'-GCCTCGATCAG(A/G)TT(A/G)TGG) (Braker, Fesefeldt, and Witzel 1998). The *nosZ* primer set was nosZ-F (5'- CG(C/T)TGTTC(A/C)TCGACA GCCAG, FAM labeled) and nosZ1622R (5'- CGC(G/A)A(C/G)GGCAA(G/C)AAGGT (G/C)CG) (Throback et al. 2004). We used Apex 2x Taq Master Mix (Genesee Scientific) for both *nirK* and *nosZ* denitrifier PCRs.

nirK is a functional gene that encodes the copper containing form of nitrite reductase, which catalyzes the first dedicated step in the denitrification pathway. Each of 33 *nirK* PCR cycles consisted of 30 seconds at 95°C, 30 seconds at 46°C, and 45 seconds at 72°C. Primers were also tested for the amplification of *nirS*, which encodes an alternate form of nitrite reductase (i.e., containing cytochrome cd_1), but repeated amplification difficulties with the primer set (nirS1F and nirS6R) (Braker, Fesefeldt, and Witzel 1998) prevented their inclusion in this study. *nosZ* is a functional gene that encodes for nitrous oxide reductase, which catalyzes the last step in the denitrification

pathway. Each of 35 *nosZ* PCR cycles consisted of 30s at 94°C, 60s at 53°C, and 60s at 72°C. Each DNA extraction (two to three per site by sampling date) was amplified in triplicate PCRs for both denitrifier primer sets.

Resulting PCR products were composited (i.e., three PCRs per extraction combined to yield two to three PCR product pools per site per date for each primer set), cleaned with Qiaquick PCR purification kits (Qiagen, Germantown, MD, U.S.A.), checked for appropriate sizes by agarose gel electrophoresis, and then used to generate terminal restriction fragment length polymorphism (TRFLP) profiles with either endonuclease Msp I for 16S rRNA products, Hae III for *nirK* products, or Mn lI for *nosZ* products. All endonucleases were from New England Biolabs (Ipswich, MA, USA). Subsequent electrophoresis runs were done with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

To process TRFLP data, we used T-REX software (Culman et al. 2009) to determine a baseline fluorescence threshold for filtering true peaks from background noise and to align terminal restriction fragments. T-REX uses a filtering algorithm that eliminates peaks that do not meet a user-specified standard deviation limit (Abdo et al. 2006). We used one standard deviation in peak area as the limit. Each peak corresponds to a terminal restriction fragment length and represents an operational taxonomic unit (OTU). Following the filtering procedure, we aligned fragments by using a clustering threshold of 0.5 base pair (Smith et al. 2005). Any OTUs present in less than 5 percent of samples were eliminated. For all subsequent analyses, we transformed the processed TRFLP data to presence-absence matrices and averaged replicates (i.e., two to three replicates for each site, date, and primer set combination) by the following logic - OTUs found present in at least one replicate were recorded as present (1), while those not found in any replicate were recorded as absent (0) at that site on that particular date.

Although TRFLP data generally underestimate the richness of highly complex communities and more advanced methods, such as cloning and sequencing, are available to describe microbial communities, we chose to use the TRFLP method because it is a cost-effective tool that has been accepted as an appropriate and efficient means of assessing overall dissimilarity among microbial community composition (Fierer et al. 2007; Fierer and Jackson 2006; Osborn, Moore, and Timmis 2000).

2.2.6 Denitrification potential

We used denitrification enzyme activity (DEA) assays to measure denitrification potentials (Tiedje 1994) of sediments collected from all four sampling dates. DEA assays are short laboratory incubations conducted at room temperature under optimal conditions (i.e., anoxia and unlimited substrate availability). Five replicate incubation slurries were prepared for each stream on each sampling date by weighing 10 g subsamples of sediment into Erlenmeyer flasks (125 mL) and adding 20 mL of stock media solution with 0.72 g potassium nitrate, 0.5 g glucose, and 0.125 g chloramphenicol in 1 L of double de-ionized water.

After adding media, flasks were topped with butyl rubber stoppers (Grace, Deerfield, Illinois, U.S.A.) to achieve airtight conditions and made anaerobic by three successive cycles of evacuation and nitrogen gas (N_2) flushing. The incubation was initiated by adding 10 mL of acetylene (C_2H_2) gas to each flask. C_2H_2 inhibits nitrous oxide (N_2O) reduction (the last step of the denitrification pathway), allowing N_2O to accumulate in the flask headspace. Flasks were continuously shaken (125 rpm) on reciprocal shakers during the 90 minute incubation. Gas samples were taken at the start of the incubation and every 30 minutes thereafter. Chloramphenicol inhibits the synthesis of new enzymes and bacterial communities are unlikely to change significantly during the short incubation period (Bernot et al. 2003).

DEA assays provide estimates of maximum denitrification rates achievable by the extant community, given optimal conditions, but without allowing sufficient time for shifts in denitrifier community structure due to growth or the synthesis of new denitrification enzymes. The rates measured by DEA assays are, therefore, a function of two key aspects of each sample: 1) the concentration of denitrification enzymes, which reflects stream conditions at the time of sampling, and 2) the structure of the denitrifier community, which reflects a legacy of stream conditions over a period of time leading up to the time of sampling. DEA assays are widely used as a valid means of comparing denitrification rates among sites (Groffman et al. 2006).

We measured N_2O concentrations using a Teledyne Tekmar 7000 headspace autosampler (Teledyne Tekmar, Mason, Ohio, U.S.A.) to inject samples into a Shimadzu GC-17A ver.3 gas chromatograph with a Porapak Q column and electron capture detector (injector temperature = 380°C, column temperature = 80°C, detector temperature = 340°C, with N_2 carrier gas). We used Bunsen coefficients to determine N_2O concentrations in each sample and calculated rates of N_2O production as the average rate observed over any 30 minute interval. N_2O production rates were then divided by the dry mass of sediments in the flask to calculate denitrification potentials (N g sediment^{-1} hr^{-1}). Statistical analyses of denitrification potentials were based on averaged potentials across all five DEA assay replicates done for each site on each sampling date.

2.2.7 Data analyses

To explore relationships among measured variables, we used R 2.11.1 (R Core Development Team) software to conduct simple linear regressions. To equalize variances and normalize residuals, denitrification potential, nitrate, TOC, and heavy

metal values were natural log transformed, while watershed % IC and % development were arcsine square root (arcsq) transformed prior to this and all other data analyses.

To test the null hypothesis of no difference in log-denitrification potential, log-nitrate, and log-TOC among streams, we used R to conduct repeated measures analysis of variance (rmANOVA) with watershed impervious cover as a factor. That is, we categorized streams *a priori* into three groups: low (<3% IC), intermediate (9 to 10% IC), and high (>10% IC) impervious cover streams. Low impervious cover streams included Mud Creek and Stony (Table 1). Intermediate impervious cover streams included Lower Mud and Pott's. High impervious cover streams included Upper Mud, Cemetery, Ellerbe, and Goose.

To test the null hypothesis of no difference in community composition among streams, we conducted permutational multivariate analysis of variance (perMANOVA) (Anderson 2001) in R using the adonis function in vegan (Oksanen et al. 2010). perMANOVA is similar to redundancy analysis (Legendre and Anderson 1999) and calculates a pseudo *F*-statistic by comparing the total variance explained by sample identity (i.e., low, intermediate, or high impervious cover) to that explained by random permutations of sample identities. We ran 9,999 permutations for each analysis. To avoid pseudoreplication, permutations were constrained by sampling date. Calculations were based on presence-absence matrices and Jaccard distance measures. A significant perMANOVA result would suggest communities sort along the urbanization gradient.

To visualize differences in community composition among sites, we created non-metric multidimensional scaling (NMS) ordinations in R using the nmds function in ecodist (Goslee and Urban 2007). We used Jaccard distance measures, random starting configurations, and 200 runs with real data for each ordination. NMS creates a mapping of samples into a reduced ordination space that preserves the rank order of ecological

distances among samples. NMS is unique in its lack of assumptions and ability to present an unbiased representation of multivariate structure in reduced space. Classical hypothesis testing can be conducted on ordination scores if test assumptions are met (Gotelli 2004; McCune and Grace 2002).

Following the ordination, we analyzed potential correlations between bacterial community composition and urbanization intensity by regressing mean ordination scores for each primer set against arcsq-transformed % IC (Urban et al. 2002).

We built a linear mixed-effects (LME) model of log-denitrification to compare the explanatory power of substrate concentration versus denitrifier community composition variables. We used LME because the method can account for non-independence of errors, such as those created by pseudoreplication, by separating explanatory variables into fixed versus random effects (Pinhiero and Bates 2002). LME analyses were done using the lme function (package nlme in R) (Pinheiro et al. 2011) with maximum likelihood estimation. We specified log-nitrate, log-TOC, and *nirK* and *nosZ* ordination scores as fixed effects and sampling date, nested within site as a random effect. We tested auto-correlation of residuals (from repeated measures) with a simple autoregressive model of order 1 (Crawley 2007). To assess model fit, we calculated a pseudo-R^2 based on a likelihood ratio test (Magee 1990).

We started with a complete model that included log-nitrate, log-TOC, all three *nirK* ordination axes scores, and all three *nosZ* ordination axes scores. Substrate concentrations were means of all available measurements from the month of each sediment sampling date. We simplified the complete model by sequentially removing the least significant terms and then using a likelihood ratio test to compare the deviance of the simpler model to that of the more complex model (Crawley 2007). If the removal led to an insignificant change in deviance, we left the term out from all further

evaluations. We also calculated the Aikaike information criterion (AIC) to compare nested models. AIC penalizes against additional parameters and decreases when more of the residual variation in log-denitrification is explained.

We built a second LME model with interaction terms to explore potential interactions between substrate concentration and denitrifier community composition. We used the same random effects structure and model simplification approach as for the first model. For the second LME model, the complete model included log-nitrate, log-TOC, and the two best performing (based on the results of the first LME model) denitrifier community composition parameters as single terms, along with all possible two-way interaction terms. We did not include more single terms and three-way interactions in the complete model because of the small size of the dataset (n = 30).

2.3 Results

2.3.1 Study stream characteristics

Study streams varied widely in terms of watershed impervious cover (0.5 to 39.4%), and watershed development (4.4 to 100%) (Table 1). Monthly water sampling over the course of this research effort also revealed a wide range of nitrate concentrations (from 0.017 to 1.706, mean: 0.327 mg NO_3-N L^{-1}) and TOC (from 1.298 to 35.525, mean: 6.350 mg C L^{-1}) concentrations across sites. Study streams also had a wide range of heavy metal concentrations (Supplementary Materials, Table 4). Total metals, a measure of cumulative heavy metal loading, ranged from 16 to 34. See Appendix A for tables of raw data.

Macroinvertebrate surveys conducted within these same study streams revealed substantial declines in the diversity of sensitive macroinvertebrate species within the families *Ephemeroptera*, *Plecoptera*, and *Trichoptera* (EPT) (Violin et al. 2011) in streams draining more highly urbanized watersheds (Sudduth et al. 2011) (Table 1 and

Supplementary Materials, Table 5). Streams with higher % IC and % development also had significantly higher total summer degree-days. Relationships between heavy metals and urbanization were significantly positive for Ni and marginally significantly positive for Al, Cd, and Pb.

2.3.2 Overall bacterial and denitrifier communities

Urbanization was not associated with a decline in microbial species richness. There were no significant correlations between watershed % IC and OTU richness (i.e., total number of OTUs per sample) for overall bacterial, *nirK* denitrifier, or *nosZ* denitrifier communities (Supplementary Materials, Table 6). While the number of species was not significantly different, microbial community composition was significantly affected by watershed urbanization. Across these eight streams, the composition of overall bacterial, *nirK* denitrifier, and *nosZ* denitrifier communities clustered into the three groups we defined based on % IC: low, intermediate, and high impervious cover streams (overall bacterial: $F_{2,11} = 1.52$, p-value = 0.032 ; *nirK* denitrifier: $F_{2,27} = 2.46$, p-value < 0.001 ; *nosZ* denitrifier: $F_{2,27} = 1.37$, p-value < 0.001).

In the NMS ordinations, communities from low and intermediate impervious cover streams were separated from communities from high impervious cover streams, as indicated by the separation along axis 1 for the overall bacterial ordination, axis 1 for the *nirK* ordination, and axis 3 for the *nosZ* ordination (Figure 2).

The linear regression of overall bacterial NMS axis 1 against arcsq-transformed % IC was highly significant ($F_{1,12} = 11.88$, $R^2 = 0.50$, p-value = 0.005) (Figure 3). The same was also true for the linear regression of *nirK* NMS axis 1 versus arcsq-transformed % IC ($F_{1,28} = 23.77$, $R^2 = 0.46$, p-value < 0.001). The linear regression of *nosZ* NMS axis 3 against arcsq-transformed % IC was also highly significant ($F_{1,28} = 49.86$, $R^2 = 0.64$, p-value < 0.001).

2.3.3 Denitrification potentials

Denitrification potentials ranged between 41 and 561 ng N g sediment^{-1} hr^{-1}, with a mean DEA of 195 ng N g sediment^{-1} hr^{-1}. Watershed land cover was not associated with significant differences in log-denitrification potentials (rmANOVA: $F_{2,3} = 1.35$, *p*-value = 0.44). Log-denitrification potentials were also not significantly different across sampling dates (rmANOVA: $F_{3,13} = 3.02$, *p*-value = 0.07).

2.3.4 Linear mixed-effects models

There was no evidence of autocorrelation of observations within groups, suggesting that errors were normally distributed within groups (i.e., sampling dates within sites). In the first LME model without interaction terms, log-nitrate, log-TOC, and all *nirK* ordination scores were removed from the final, best fitting model (Table 2 and Supplementary Materials, Table 7). The final model included all three *nosZ* ordination scores and captured an estimated 38% of the variation in log-denitrification. Although nosZ ordination axis 3 was not a significant term, its removal increased AIC and decreased pseudo-R^2, so we kept the term. Compared to the intercept only model (AIC = 69.51), the final model (AIC = 61.21) had lower deviance (*p*-value = 0.003).

In the second LME model with two-way interaction terms, the starting model included log-nitrate, log-TOC, and nosZ ordination axes 1 and 2, along with all two-way interactions. We used ordination axes 1 and 2 because they explain a larger proportion of the variation in composition than any other combination of axes. Log-nitrate, log-TOC, and all two-way interactions, except that between log-TOC and *nosZ* ordination axis 1, were deleted from the final, best fitting model (Table 3 and Supplementary Materials, Table 8). The final model (AIC = 57.62) captured an estimated 45% of the variation in log-denitrification and had lower deviance than the intercept only model (AIC = 69.51) (*p*-value < 0.001).

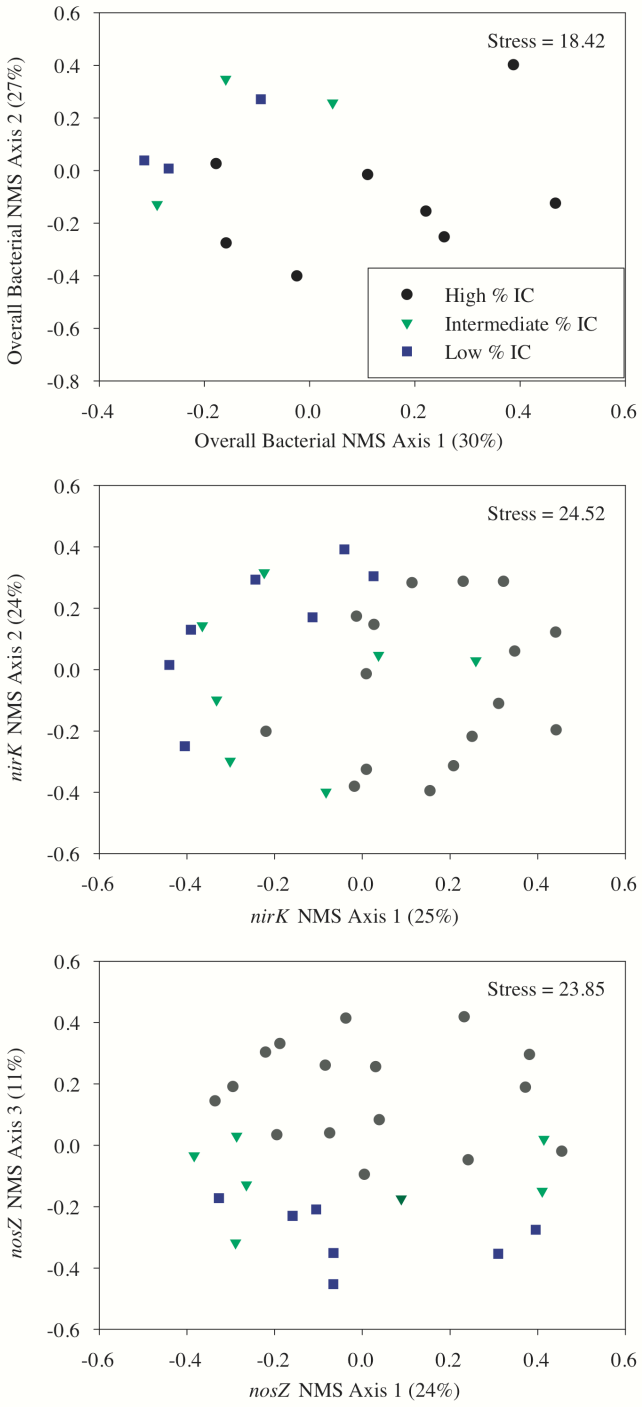

Figure 2: Non-metric multidimensional scaling (NMS) ordination of overall bacterial and *nirK* and *nosZ* denitrifier communities

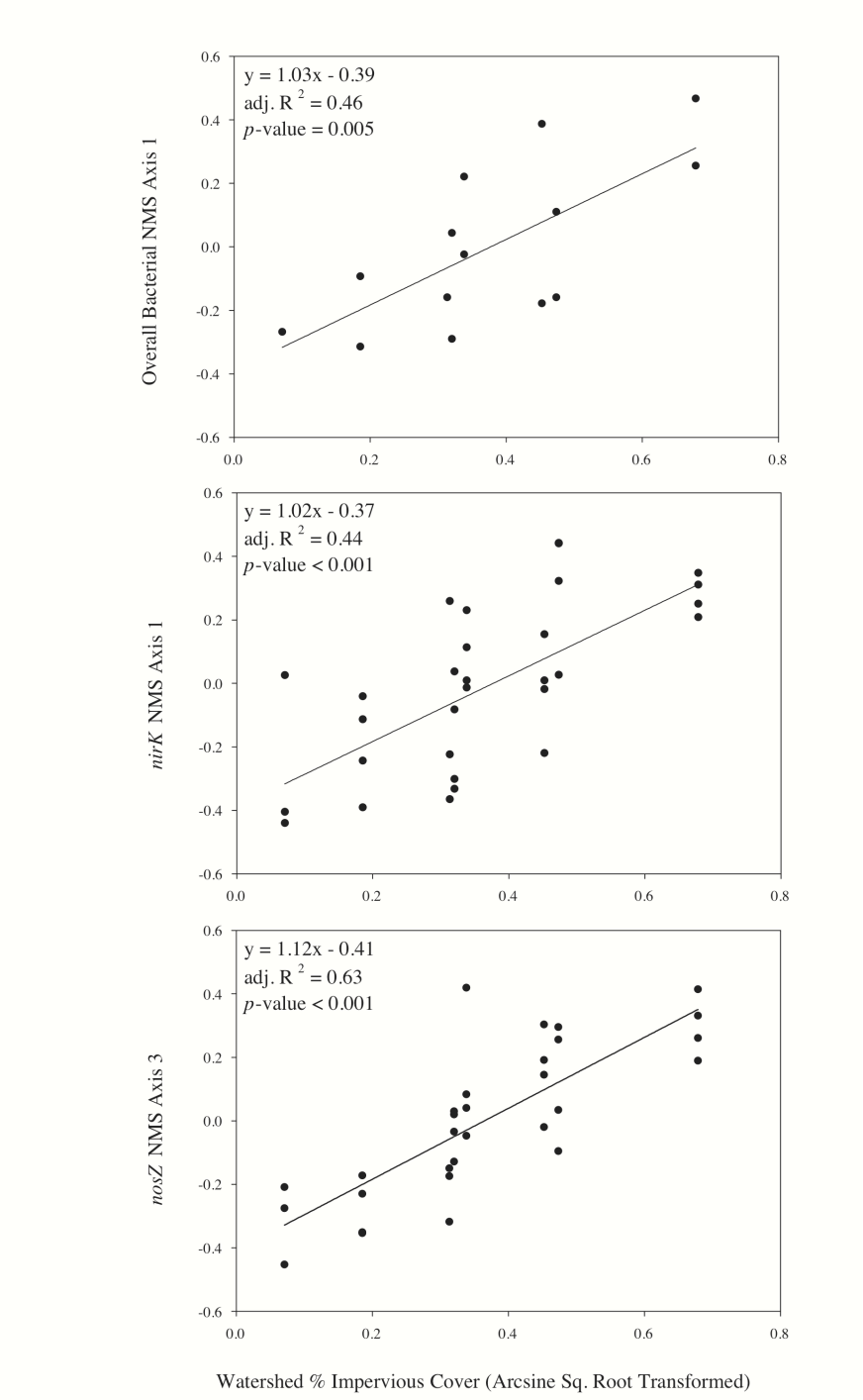

Figure 3: Linear regressions of ordination axes scores against arcsine square root transformed percent impervious cover.

Table 2: Results from ANOVA conducted to determine significance of fixed effects of the final linear mixed-effects model (without interactions) of log-denitrification

Parameter	Num DF	Den DF	*F*-value	*p*-value
Intercept	1	19	956.38	< 0.001
nosZ ordination axis 1	1	19	7.875	0.011
nosZ ordination axis 2	1	19	4.636	0.044
nosZ ordination axis 3	1	19	3.403	0.081

Note: While *nosZ* ordination axis 3 was not a significant term, its removal increased AIC (see Supplementary Table 3) and decreased pseudo-R^2, so we kept the term in the final model.

Table 3: Results from ANOVA conducted to determine significance of fixed effects of the final linear mixed-effects model (with interactions) of log-denitrification

Parameter	Num DF	Den DF	*F*-value	*p*-value
Intercept	1	19	796.17	< 0.001
nosZ ordination axis 1	1	19	9.420	0.006
nosZ ordination axis 2	1	19	5.899	0.025
nosZ ordination axis 1: log-TOC	1	19	6.977	0.016

2.4 Discussion

Watershed urbanization imposes numerous stressors on stream inhabitants, including increased contaminant concentrations, streamwater temperatures, and hydrologic disturbance (Walsh et al. 2005). Our results suggest that these urban stressors can drive changes in the composition of bacterial communities in streams. Given the key roles bacteria play in the biogeochemical cycling of nutrients and organic matter (Falkowski, Fenchel, and Delong 2008), such compositional changes could affect

ecosystem functioning, particularly if the composition or activity of specific functional groups within the overall bacterial community are altered by urban inputs or stressors. In our study, denitrifier communities sorted along the urbanization gradient, regardless of whether nitrite (*nirK*) or nitrous oxide (*nosZ*) reductase primers were used to characterize communities. Knowledge of denitrifier community composition, in turn, greatly improved our ability to capture observed variations in denitrification potential.

2.4.1 Watershed %IC is a good predictor of bacterial community composition in streams

Our study streams encompassed chemical (nutrients, heavy metals), physical (streamwater temperatures) and hydrologic (flashiness) gradients that were generally positively associated, though not always significantly, with watershed % IC. While several factors are likely to regulate bacterial community composition, we found that a single variable, watershed % IC, was strongly and significantly correlated with overall bacterial and denitrifier community composition across our study streams. Moreover, communities from low, intermediate, and high % IC streams were significantly different from one another.

Clearly, watershed land cover itself is not directly driving differences in bacterial community composition. Rather, % IC provides an integrative measure of the intensity with which watershed urbanization may be altering numerous different measured and unmeasured aspects of stream conditions, which are, in turn, regulating bacterial community composition. Given the limited number of sites in this study, we cannot identify the specific urban impacts primarily responsible for observed patterns in bacterial community composition. We can, however, conclude that watershed % IC is a good predictor of bacterial community composition in these streams and that watershed

urbanization has the potential to strongly affect bacterial community composition, albeit through as yet unidentified pathways.

Further support for these conclusions can be found in the consistency with which the composition of different groups of bacteria (i.e., overall bacteria, *nirK* denitrifiers, and *nosZ* denitrifiers) appeared to sort along the urbanization gradient identified in this study. In contrast, TRFLP estimates of OTU richness for all three groups did not exhibit any obvious association with watershed % IC, perhaps suggesting that bacterial community richness was generally insensitive to urban impacts in these streams.

2.4.2 Denitrifier community composition helps explain denitrification potential

Model results indicate that denitrifier community composition may be more important than substrate supply in driving denitrification rates in our study streams. The first LME model captured an estimated 38% of the variation in denitrification potential among streams and included three composition parameters, but no substrate parameters. The second LME model captured an estimated 45% of the variation in denitrification potential among streams and included two composition parameters and an interaction term between composition and organic carbon. The interaction suggests that the capacity of communities to utilize available carbon substrates depends, in part, on which denitrifier taxa are present.

These results suggest that one important pathway through which watershed urbanization may alter stream ecosystem functioning is by changing bacterial community composition in streams. That is, community composition can directly influence rates of functioning, independent of environmental factors. It remains unclear whether the observed links between urbanization-driven shifts in denitrifier community composition and denitrification potential extend to other microbe-mediated ecosystem

processes in streams, such as decomposition and carbon cycling. Future studies could address this research gap by characterizing different functional groups of bacteria and measuring their function rates in streams with varying degrees of watershed urbanization.

2.4.3 Conclusions

Microbes are the most ubiquitous, abundant, and diverse group of organisms on Earth (Pace 1997). Our understanding of how ecosystems respond to land-use change would be incomplete without the microbial perspective. Ecologists generally agree that shifts in plant community composition can alter ecosystem process rates (Hector et al. 2011; Loreau et al. 2001). The same could be true for microbial community composition, yet ecosystem modeling efforts have largely ignored microbial community composition (Allison and Martiny 2008). Given advances in molecular technology, we can now question the assumption that microbial communities are resistant to anthropogenic disturbances and that they can be adequately represented as 'black boxes' responding only to substrate supply in ecosystem models (Zak, Blackwood, and Waldrop 2006).

This study demonstrates that incorporating data on bacterial community composition, even relatively low resolution data from a molecular fingerprinting method, can drastically improve our ability to model ecosystem process rates, particularly under realistic scenarios of environmental degradation. To identify and rank specific drivers of bacterial community change in urban streams, we need to survey a larger number of streams and collect more comprehensive data on factors like contamination, temperatures, and hydrology. This information would improve our ability to understand the detrimental impacts of urbanization on microbial community structure and function. Future research should focus on understanding not only the

causes of microbial community change in human impacted ecosystems, but also how these communities differ in resistance and function.

2.5 Supplementary Materials

Table 4: Heavy metals concentrations in stream sediments

Stream	Ag	Al	As	Cd	Cr	Cu	Ni	Pb	Zn
Mud Creek	0.148	29.51	0.646	0.022	3.410	6.057	1.978	3.866	18.25
Stony	0.013	29.19	1.781	0.043	8.367	108.6	1.391	7.629	67.26
Lower Mud	0.017	24.81	1.492	0.029	12.01	7.152	3.745	4.855	20.18
Pott's	0.029	45.83	0.417	0.025	17.41	90.38	2.611	10.25	37.35
Upper Mud	0.297	44.00	1.660	0.030	14.75	9.278	5.117	8.064	23.76
Cemetery	0.025	48.73	0.263	0.040	6.049	55.18	3.406	28.07	44.61
Ellerbee	0.146	35.83	0.470	0.028	12.37	5.652	5.477	5.740	14.00
Goose	0.041	50.90	0.858	0.054	8.218	11.57	7.913	13.89	36.06

Notes: All values are given in $\mu g\ g^{-1}$ ash free dry mass sediment, except for Al. Al values are given in $mg\ g^{-1}$ ash free dry mass sediment.

Table 5: Correlations (Pearson's *r*) between watershed metrics and measured stream characteristics across all sites and dates

	Watershed impervious cover	Watershed development
Watershed impervious cover		
Watershed development	**0.940** $^{<0.001}$	
Nitrate	0.370	0.609
TOC	0.509	0.303
Ag	-0.035	-0.014
Al	**0.669** $^{0.070}$	0.600
As	-0.260	-0.261
Cd	0.617	**0.644** $^{0.085}$
Cr	0.311	0.179
Cu	-0.106	-0.144
Ni	**0.856** $^{0.007}$	**0.817** $^{0.013}$
Pb	0.594	**0.647** $^{0.083}$
Zn	0.036	0.024
Total metals	0.605	0.557
Degree-days	**0.894** $^{0.003}$	**0.825** $^{0.012}$
Flashiness	0.526	0.588
EPT richness	**-0.796** $^{0.018}$	**-0.870** $^{0.005}$

Notes: Watershed metrics were arcsine square root transformed and nitrate, TOC, and heavy metal concentrations were natural log transformed prior to analysis. Values in bold indicate correlations with *p*-values < 0.1 (given in superscript).

Table 6: Correlations (Pearson's *r*) between watershed metrics and bacterial OTU richness across all sites and dates

	Watershed impervious cover	Watershed development
Watershed impervious cover		
Watershed development	**0.940** $^{<0.001}$	
Overall bacterial OTU richness	-0.050	-0.170
nirK denitrifier OTU richness	-0.196	-0.254
nosZ denitrifier OTU richness	0.525	0.683

Notes: Watershed metrics were arcsine square root transformed prior to analysis. Values in bold indicate correlations with *p*-values < 0.1 (exact values given in superscript).

Table 7: Non-significant terms deleted from the complete version of the first linear mixed-effects model of log-denitrification (without interaction terms)

Model	Parameters	df	AIC	Log-likelihood	Likelihood ratio	*p*-value
1	log-nitrate, log-TOC, nir1, nir2, nir3, nos1, nos2, nos3	2	9.27	-22.64		
2	log-nitrate deleted	1	8.20	-23.10	0.92	0.336
3	nir1 deleted	0	6.44	-23.22	0.24	0.626
4	nir3 deleted	9	4.79	-23.40	0.35	0.552
5	nir2 deleted	8	3.06	-23.53	0.27	0.601
6	log-TOC deleted	7	1.21	-23.60	0.14	0.705
7	nos3 deleted	6	2.69	-25.34	3.48	0.062

Notes: The parameters nir1, nir2, and nir3 refer to scores for *nirK* ordination axes 1, 2, 3, respectively. The parameters nos1, nos2, and nos3 refer to scores for *nosZ* ordination axes 1, 2, 3, respectively. Likelihood ratios and *p*-values refer to the change in deviance that resulted from the deletion of each term from the more complex model in the row above.

Table 8: Non-significant terms deleted from the complete version of the first linear mixed-effects model of log-denitrification (with interaction terms)

Model	Parameters	df	AIC	Log-likelihood	Likelihood ratio	*p*-value
1	log-nitrate, log-TOC, nos1, nos2, and all two-way interactions	14	68.98	-20.49		
2	log-nitrate : nos2 deleted	13	66.99	-20.50	0.01	0.912
3	nos1 : nos2 deleted	12	65.04	-20.52	0.04	0.837
4	log-nitrate : nos1 deleted	11	63.05	-20.52	0.01	0.908
5	log-nitrate : nos2 deleted	10	61.29	-20.65	0.24	0.623
6	log-nitrate deleted	9	61.17	-21.58	1.88	0.170
7	log-TOC deleted	8	59.51	-21.76	0.34	0.559
8	log-TOC : log-nitrate deleted	7	57.62	-21.81	0.11	0.741
9	nos2	6	62.65	-25.33	7.03	0.008

Notes: The parameters nir1, nir2, and nir3 refer to scores for *nirK* ordination axes 1, 2, 3, respectively. The parameters nos1, nos2, and nos3 refer to scores for *nosZ* ordination axes 1, 2, 3, respectively. Likelihood ratios and *p*-values refer to the change in deviance that resulted from the deletion of each term from the more complex model in the row above.

3. Bacterial diversity is linked to higher functional resistance

3.1 Introduction

As humans continue to alter ecosystems through land use change and climate change, there is a growing need to better understand and predict how biotic communities might respond to increasingly compounded and severe disturbance (Paine, Tegner, and Johnson 1998). One key element of a community's response is its degree of functional resistance, defined here as the degree of change in community function caused by a stress or disturbance. Functional resistance can be strongly affected by community structure (McCann 2000). Given the key role microorganisms play in mediating many valuable ecosystem services (Falkowski, Fenchel, and Delong 2008), the need to better understand how microbial communities might respond to anthropogenic disturbance is particularly acute (Allison and Martiny 2008). While there are many competing hypotheses for how community structure might be linked to resistance for plants and animals (i.e., macroorganisms), there have been relatively few tests of these hypotheses for naturally occurring microbial communities.

This study compares the functional resistance of two naturally occurring communities of denitrifying bacteria (denitrifiers) to experimental disturbances in laboratory microcosms. The objective was to determine whether communities from a highly disturbed environment were more resistant (legacy hypothesis) or, assuming that the more disturbed environment had lower diversity, less resistant (insurance hypothesis) than communities from a relatively pristine environment. Microcosms were assembled with experimental communities from either an urban stream or a forest stream. We focused on denitrifier communities because they are a phylogenetically diverse functional group whose function (denitrification) is ecologically important and

easily quantified (Peterson et al. 2001; Philippot and Hallin 2005). The disturbances tested were increased pollution and increased temperature in a fully factorial experimental design.

Theoretically, communities composed largely of tolerant taxa should be highly resistant. A drought, for instance, will not cause large changes in function for a drought-adapted community dominated by drought tolerant taxa. Thus, the historic disturbance regime and its selective impact on community structure can play a key role in determining resistance. We refer to this theory as the legacy hypothesis, which predicts high resistance for communities that have had previous experience with that type of disturbance (White 1979). Community diversity (i.e., richness and evenness) can also affect resistance. According to the insurance hypothesis, communities with high diversity are more likely to have members that can respond favorably to a disturbance and, therefore, maintain high rates of functioning (Yachi and Loreau 1999). Communities with high diversity are also more likely to have high redundancy. That is, diverse communities are more likely to have members that can functionally replace important members lost to disturbance (Naeem and Li 1997).

Urban streams are typically more polluted and warmer than forest streams (Walsh et al. 2005). We might therefore predict that urban denitrifier communities would be accustomed to these disturbances and be relatively insensitive to pollutant exposure or warming (legacy hypothesis). Alternatively, urban denitrifier communities may already be under such strong selection that they have reduced diversity and, therefore, a reduced capacity to respond to disturbances (insurance hypothesis). There is some support for the latter hypothesis, since ecologists have found that urbanization can lead to reduced diversity for stream macroorganisms (Roy et al. 2009). Microbes, however, have exceedingly high diversity relative to macroorganisms, an attribute that

some have argued make all microbial communities highly resistant (Allison and Martiny 2008).

3.2 Methods

3.2.1 Community origins

The experimental communities orginated from Goose Creek and Mud Creek, two streams with contrasting watershed land covers in the Raleigh-Durham metropolitan area of the Piedmont region in North Carolina (USA) (Table 9). We selected the streams to represent the endpoints of a gradient of urbanization intensity in the area. Goose Creek drains a watershed with 39% impervious cover. Mud Creek drains a watershed dominated by protected, mature hardwood trees and has less than 1% impervious cover.

Both streams have been monitored for streamwater quality (e.g., nitrogen, carbon, chloride, etc.) and temperatures since 2006 (Sudduth et al. 2011; Violin et al. 2011). Streamwater samples are taken biweekly and temperatures are recorded every ten minutes with sondes (model 600XLM, YSI, Yellow Springs, OH, USA). We measured streamwater quality and temperatures, as well as sediment heavy metal concentrations (described below), because one assumption behind the legacy hypothesis is that communities from the urban stream have had previous experience in the field with the disturbances tested in this study, which included two pollution treatments and an increased temperature treatment.

On June 02, 2009, we collected sediments from streams for heavy metals analyses by randomly selecting five points along a permanently established study reach (~100 m) at each site. At each selected point, we demarcated a 2 m segment of the streambed (i.e., entire wetted width) upstream and downstream of the point and used PVC corers (6.35 cm diameter) to take multiple sediment cores until a total volume of at least 4,024 cm^3 was collected. Sediment cores were sieved (2 mm opening) and composited, resulting in

a single composited sediment sample from each site. Sediment samples were kept on ice for transport to the laboratory and stored at 4°C prior to analyses.

Table 9: Study stream characteristics

	Forest Stream (Mud Creek)	Urban Stream (Goose Creek)
Impervious cover (%) [1]	0.5	39.4
Development (%) [1]	4.4	100
Nitrate (mg L^{-1}) [2]	0.11	0.20
TOC (mg L^{-1}) [2]	4.78	15.17
Chloride (mg L^{-1}) [2]	14.12	19.04
Total metals (μg g^{-1}) [3]	148	169
EPT richness [4]	4	0
Temperature (°C) [5]	19.29	22.47
Flashiness [6]	0.04	0.17

Notes:
[1] Watershed land cover values calculated using 2005 land use/land cover imagery. NLCD classes 22, 23, and 24 were classified as developed areas
[2] Mean streamwater concentrations recorded between June 2008 and July 2009
[3] Sum of concentrations for nine heavy metals (Ag, Al, As, Cd, Cr, Cu, Ni, Pb, and Zn) in sediment samples from June 02, 2009
[4] Mean number of macroinvertebrate species belonging to *Ephemeroptera*, *Plecoptera*, and *Trichoptera* found in 2006 and 2007 surveys (Violin et al. 2011). EPT richness is often used as an indicator of water quality; EPT species tend to occur only in clean, well oxygenated waters (Rosenberg and Resh 1993).
[5] Mean streamwater temperatures recorded between May 20, 2009 and June 10, 2009
[6] Flashiness estimated from changes in hourly discharge between May 2007 and June 2007 (Sudduth et al. 2011)

We measured the concentrations of nine heavy metals (Ag, Al, As, Cd, Cr, Cu, Ni, Pb, Zn) in sediment samples by re-sieving (1 mm opening), drying at 60°C for 48 hours, and then weighing out three replicate 1 g subsamples for each site on each date. Subsamples were digested by adding 10 mL of 50% nitric acid, heating at 95°C, and then adding another 5 mL of nitric acid to each subsample. We then used inductively coupled plasma-mass spectrometry (Perkin-Elmer Elan 6000, Perkin-Elmer, Waltham, MA, USA) to measure metals concentrations. Three replicates of certified reference material STSD-3

(NRC, Institute for National Measurement Standards, Ottawa, Canada) and two method blanks were processed for every 35 samples analyzed.

3.2.2 Microcosm assembly

On April 30, 2009, we collected sediments from streams using the same collection method as for the heavy metals analyses. Sediment samples were kept on ice for transport to the laboratory and stored at 4°C for less than 3 hours before being used to make inocula for the microcosms. We weighed 60 g of sediment per stream into sterile tubes, added 450 mL of autoclaved spring water, vortexed at maximum power for 5 minutes, and then passed the resulting mixtures through sterile Whatman GF/F filters (Whatman International Ltd., Kent, UK) to create two types of inocula – an urban community and a forest community inoculum. Inocula were then kept at 4°C overnight before being added to microcosms the next day.

Before adding inocula to microcosms, we first estimated the active microbial biomass in each type of inoculum to determine the approximate volumes to add in order to achieve similar biomass quantities in all microcosms. Active microbial biomass was estimated using a modified (Fierer, Schimel, and Holden 2003) substrate induced respiration (SIR) method (West and Sparling 1986) using autolyzed yeast extract as the substrate. Briefly, the method involved amending inocula samples (5 replicates for each type) with substrate solution before measuring CO_2 production rates over 4.5 hours. Given an essentially unlimited substrate supply and a short incubation, CO_2 production rates should be directly proportional to the active microbial biomass in sediment samples (Johansson, Pell, and Stenstrom 1998).

We assembled microcosms by adding either 50 uL of urban or 65 uL of forest community inoculum to sterile media (trypticase soy broth) spiked with 2 mM nitrite in multiwell culture plates (Corning Costar 3370; Corning Life Sciences, Lowell, MA, USA).

We added 30% more inoculum into forest versus urban wells because SIR results suggested that the urban inoculum had approximately 30% greater active microbial biomass than the forest inoculum. Mean CO_2 production rates were 0.0375 ppm CO_2 $mL^{-1} hr^{-1}$ for the urban and 0.0289 ppm CO_2 $mL^{-1} hr^{-1}$ for the forest community inoculum. Nitrite concentrations in both inoculum types were below detection (< 0.03 mM). Total volume in each well was 250 μL.

3.2.3 Experimental design

A total of 715 experimental microcosms were assembled. 360 were inoculated with forest inoculum and 355 were inoculated with urban inoculum. To test community responses to disturbance, microcosms were incubated anaerobically for 20hrs with or without pollutants (0.002 ppm $AgNO_3$ (silver) alone, 0.26 ppm NaCl (salt) alone, or a mixture of 0.002 ppm silver and 0.26 ppm salt) at either 28 or 38°C (Table 10). While the addition of $AgNO_3$ also adds nitrate, an important denitrification substrate, the concentrations added (7.3×10^{-4} ppm nitrate) are orders of magnitude less than nitrite concentrations in the media (51×10^{4} ppm nitrite) and are unlikely to lead to additional stimulation of denitrification rates.

Table 10: Experimental treatments and replication levels

	28°C	38°C	Total
No Pollution	46 / 46	39 / 38	85 / 84
$AgNO_3$ alone	47 / 48	46 / 46	93 / 94
NaCl alone	47 / 48	47 / 46	94 / 94
$AgNO_3$ + NaCl	44 / 46	39 / 42	83 / 88
Total	184 / 188	171 / 172	355 / 360

Notes: Values refer to the number of microcosms inoculated with urban (left of slash) or forest (right of slash) inoculum and incubated under the specified treatment conditions.

We chose to test silver and salt because both chemicals have been found in high concentrations in urban streams (Herlihy, Stoddard, and Johnson 1998; Neal and Robson 2000) and both chemicals are known to affect denitrification rates (Seo, Yu, and Delaune 2008; Throback et al. 2007). We chose to test a 10°C temperature increase because previous measurements have documented differences of as much as 15°C between the two streams in the summer season (Sudduth et al. 2011) (Figure 4). Temperatures are known to strongly affect denitrification rates (Stanford, Dzienia, and Vander Pol 1975).

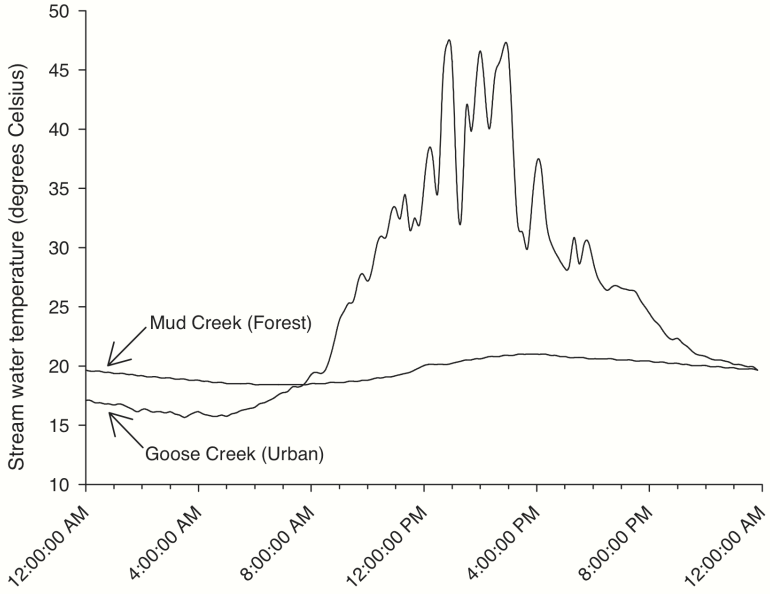

Figure 4: Streamwater temperatures in study streams over a 24-hour period (June 07, 2009) in the summer season

Microcosms were made anaerobic by placing them in airtight chambers with gas generating sachets that create an anaerobic atmosphere suitable for culturing anaerobic bacteria (BD GasPak EZ Container System, BD Diagnostics, Franklin Lakes, NJ, USA). We used indicator strips to check that all chambers reached and maintained anaerobic conditions.

As with Wittebolle et al. (2009), we estimated net ecosystem denitrification of the microbial communities by calculating the difference between the mean nitrite concentration of the negative controls (i.e., no inocula added) and the final nitrite concentration in each inoculated microcosm at the end of the incubation. Final nitrite concentrations were quantified spectrophotometrically on a plate reader (BMG Labtech, Cary, NC, USA).

3.2.4 Characterizing denitrifier communities

To explore potential links between initial community diversity and resistance, we used terminal restriction fragment length polymorphism (TRFLP) profiles to estimate operational taxonomic unit (OTU) richness and evenness. While communities may have changed in structure during the course of the experiment, our objective was to test the functional response of the initial community to treatments, not to examine any potential community shifts. We focused, therefore, on characterizing the initial denitrifier communities in samples collected from the study streams.

We extracted DNA with PowerSoil kits (MoBio Laboratories, Carlsbad, CA, USA) from stream sediments collected on April 30, 2009 for making inocula. Extractions were done in triplicate for each stream. Following extraction, PCRs with *nirK* primers (FAM labeled nirK1F and nirK5R) (Braker, Fesefeldt, and Witzel 1998) and Taq Master Mix (Sigma-Aldrich, St. Louis, MO, USA) were used to amplify denitrifier DNA. *nirK* is a functional gene that encodes the copper containing form of nitrite reductase, a key enzyme in the denitrification pathway. Each of 33 *nirK* PCR cycles consisted of 30 seconds at 95°C, 30 seconds at 46°C, and 45 seconds at 72°C. Three separate *nirK* PCRs were done for each DNA extraction. There is another form of nitrite reductase encoded by *nirS*, but we were unable to amplify *nirS* genes (using primer set nirS1F and nirS6R) (Braker, Fesefeldt, and Witzel 1998) from these samples.

Products from the three PCRs per extraction were combined to yield one product pool per extraction. Since three extractions were done per stream, this ultimately yielded three PCR product pools per stream. PCR product pools were then cleaned with Qiaquick PCR purification kits (Qiagen, Germantown, MD, USA), checked for appropriate sizes by agarose gel electrophoresis, and then used to generate TRFLP products with endonuclease Hae III (New England Biolabs, Ipswich, MA, USA). Subsequent electrophoresis runs were done with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

To process TRFLP data, we used T-REX software (Culman et al. 2009) to determine a baseline threshold for filtering true peaks from background noise. Each peak corresponds to a unique restriction fragment length and represents an OTU. After filtering, we aligned peaks using a clustering threshold of one base pair. Samples with less than five peaks were eliminated. We transformed TRFLP data to relativized abundances by standardizing each peak area by the within-sample total peak area for all further analyses.

3.2.5 Data analyses

To test whether streamwater chloride and sediment heavy metal concentrations were higher at the urban versus forest stream, we conducted one-tailed Student's *t*-tests with measurements made on streamwater samples collected on April 28, 2009 and on sediment samples collected on June 02, 2009. All analyses were done in R (v2.11.1, R Core Development Team). We tested whether daily mean streamwater temperatures were different between streams with a one-way repeated measures analysis of variance (rmANOVA) with fixed factors (i.e., urban or forest). We used data from May 20, 2009 to June 10, 2009 because the urban stream sonde was malfunctioning in April (and most of May) 2009.

To determine whether denitrifier community OTU richness and evenness were lower for the urban versus forest denitrifier communities, we conducted one-tailed Student's *t*-tests using data from *nirK* TRFLP profiles. OTU richness is simply the total number of restriction fragment lengths identified in a TRFLP sample, while OTU evenness was quantified by calculating the Gini coefficient (G), which ranges from zero to one. A value of zero represents a perfectly even community, while values close to one indicate a highly uneven community dominated by very few taxa (Naeem 2009; Wittebolle et al. 2009). We used the ineq function (in package ineq) to calculate G values for each TRFLP sample.

Besides richness and evenness, urban and forest denitrifier communities may have also differed in terms of composition (i.e., species identity). To explore this possibility, we tested the null hypothesis of no difference in community composition between study streams by using the adonis function in vegan (Oksanen et al. 2010) to conduct a permutational multivariate analysis of variance (perMANOVA) (Anderson 2001). perMANVOA is similar to redundancy analysis (Legendre and Anderson 1999) and calculates a pseudo *F*-statistic by comparing the total variance explained by sample identity (in this case, urban versus forest stream) to that explained by random permutations (n = 9999) of sample identities. We used relativized abundances and Bray-Curtis distance measures.

To assess the effect of treatments on denitrification, we conducted a three-way analysis of variance (ANOVA) with fixed factors, including community type (urban or forest), pollution treatment (none, $AgNO_3$ alone, NaCl alone, or combined $AgNO_3$ and NaCl), and temperature treatment (28 or 38°C). Following ANOVA, we compared mean denitrification between reference (i.e., no pollution and 28°C) and treated microcosms *within* the same community type. To adjust for multiple comparisons, we used the

Tukey Honest Significant Differences (Tukey 1949) method with a family wise confidence level of 0.95.

3.2.6 Experimental caveats

April streamwater temperatures are generally around 14°C, so microcosms incubated at 28°C with no pollution treatments (i.e., reference microcosms) likely already represent a temperature increase from field conditions. We chose to use 28°C as the baseline incubation temperature because standard microbiology culturing protocols typically use temperatures between 20 and 38°C (Heylen et al. 2006). Despite this caveat, the experimental design still allows robust estimates of functional resistance associated with each community type. The key metric is the difference in net denitrification between reference and treated microcosms *within* each community type, not across community types. As long as all urban reference microcosms, for instance, experience comparable levels of disturbance associated with the microcosm setup itself, we can establish a reference urban rate against which to compare rates for other urban microcosms under treatment stress. Large differences between reference and stressed rates indicate low resistance for that community type and treatment.

Uncontrolled abiotic differences (e.g., salinity, organic matter, nutrients, pH, etc.) between urban and forest stream sediments used to make the inocula are potentially important confounding factors in this experimental design. To address this issue, we filtered and twice diluted inocula with spring water and trypticase soy broth, actions that should have reduced concentrations of chemicals that may have carried over into the inocula from sediments. Moreover, since the media used in all microcosms had high nutrient concentrations, any potential differences in nutrient concentrations between urban and forest inoculum should have been overwhelmed by the theoretically unlimited supply in the media.

Since estimates of functional resistance were based on differences between reference and treated microcosms within the same community type, differences between urban and forest stream inocula would not have affected our interpretations of whether a particular community type was resistant or sensitive to a treatment. For example, as long as all urban microcosms had similar pH, we can use differences in net denitrification between reference and treated microcosms to determine functional resistance for urban communities.

Comparisons between communities, however, will be affected by any substantial abiotic and biotic (e.g., other non-denitrifying bacteria) differences between inocula. While the added complexity may make our experimental results more difficult to interpret relative to traditional, culture-based experiments that only include known denitrifier species, we argue that our approach is a step in the right direction towards a more realistic assessment of how highly complex microbial communities might respond to additional stressors in environments with differing degrees of pre-existing disturbance.

3.3 Results

Streamwater chloride concentrations were higher in the urban (mean = 31.0 mg L^{-1}) versus forest (mean = 13.0 mg L^{-1}) stream (Student's *t*-test, one-tailed: $p = 0.002$). Eight heavy metals had higher concentrations in urban versus forest stream sediments, including Al, As, Cd, Cr, Cu, Ni, Pb, and Zn (Student's *t* test, one-tailed: $p < 0.05$) (Table 11). Ag concentrations were comparable between streams (Student's *t* test, one-tailed: $p = 0.751$). Daily mean streamwater temperatures were higher in the urban (mean = 22.5°C) versus forest (mean = 19.3°C) stream (rmANOVA: $F_{1,21} = 100.8$, $p < 0.001$).

Table 11: Student *t*-tests (one-tailed) for heavy metal concentrations

Heavy metal	Forest stream mean conc. (μg g^{-1} sediment)	Urban stream mean conc. (μg g^{-1} sediment)	df	*t*	*p*-value
Ag	0.19 (0.29)	0.05 (0.03)	2.03	0.817	0.751
Al	37.17 (0.85)	59.67 (2.31)	2.53	-15.84	< 0.001*
As	0.81 (0.11)	1.00 (0.05)	2.67	-2.71	0.042*
Cd	0.03 (0.00)	0.06 (0.00)	3.20	-53.5	< 0.001*
Cr	4.30 (0.62)	9.60 (0.20)	2.41	-14.00	< 0.001*
Cu	7.63 (0.84)	13.53 (0.75)	3.95	-9.08	< 0.001*
Ni	2.50 (0.35)	9.23 (1.01)	2.46	-10.91	0.002*
Pb	4.87 (0.21)	16.23 (1.60)	2.07	-12.17	0.003*
Zn	23.03 (0.95)	42.13 (3.12)	2.37	-10.14	0.003*

Notes: One-tailed Student's *t*-tests for higher heavy metal concentrations in urban versus forest stream sediments. Standard deviations in parentheses.

OTU richness tended to be higher for forest (mean = 44 OTUs) versus urban (mean = 16 OTUs) denitrifier communities, although the difference was only marginally significant (Student's *t* test, one-tailed: $p = 0.053$). Forest communities (mean G value = 0.63) were significantly more even than urban communities (mean G value = 0.87) (Student's *t* test, one-tailed: $p = 0.021$). Denitrifier community composition was not significantly different between urban and forest communities (perMANOVA: $F_{1,3} = 7.457$, $p = 0.099$). See Appendix B for tables of raw data.

ANOVA results indicated that all pollution and temperature treatments had significant effects on net denitrification (Table 12). There was a significant interaction between community type (i.e., urban or forest stream) and pollution treatment ($F_{3,699} = 8.951$, $p < 0.001$). In contrast, there was no significant interaction between community type and temperature treatment ($F_{1,699} = 0.119$, $p = 0.730$). The interaction between temperature and pollution treatment was significant ($F_{3,699} = 3.585$, $p = 0.014$).

Table 12: Results of three-way ANOVA with fixed factors for net denitrification

	df	Sum of squares	Mean square	F-ratio	*p*-value
Stream	1	1.106	1.106	9.342	0.002*
Pollution	3	12.065	4.022	33.961	< 0.001*
Temperature	1	3.589	3.589	30.306	< 0.001*
Stream : Pollution	3	3.179	1.060	8.948	< 0.001*
Stream : Temperature	1	0.015	0.015	0.128	0.721
Pollution : Temperature	3	1.274	0.425	3.585	0.014*
Stream : Pollution : Temperature	3	0.099	0.033	0.278	0.841
Within groups (residual)	699	82.772	0.118		

Net denitrification for forest communities was unaffected by the silver treatment (adj. $p = 0.993$) and stimulated by the salt treatment (adj. $p < 0.001$) (Figure 5). The combined silver and salt treatment did not affect net denitrification for forest communities (adj. $p = 0.999$). Net denitrification for urban communities was unaffected by the silver (adj. $p = 0.998$) and stimulated by the salt (adj. $p < 0.001$) treatment. Unlike the forest community, the combined silver and salt treatment reduced net denitrification for urban communities (adj. $p = 0.043$).

Net denitrification for forest and urban communities were unaffected by the temperature treatment (adj. $p = 0.915$ and 0.999, respectively). Net denitrification for forest communities given the temperature treatment together with the silver, salt, or combined silver and salt treatment were unaffected (adj. $p = 1.000$, 0.995, and 1.000, respectively). Net denitrification for urban communities given the temperature treatment together with the silver or salt treatment were also unaffected (adj. $p = 1.000$ and 1.000, respectively). When given the temperature treatment and the combined silver and salt treatment, net denitrification for urban communities was significantly reduced (adj. $p < 0.001$).

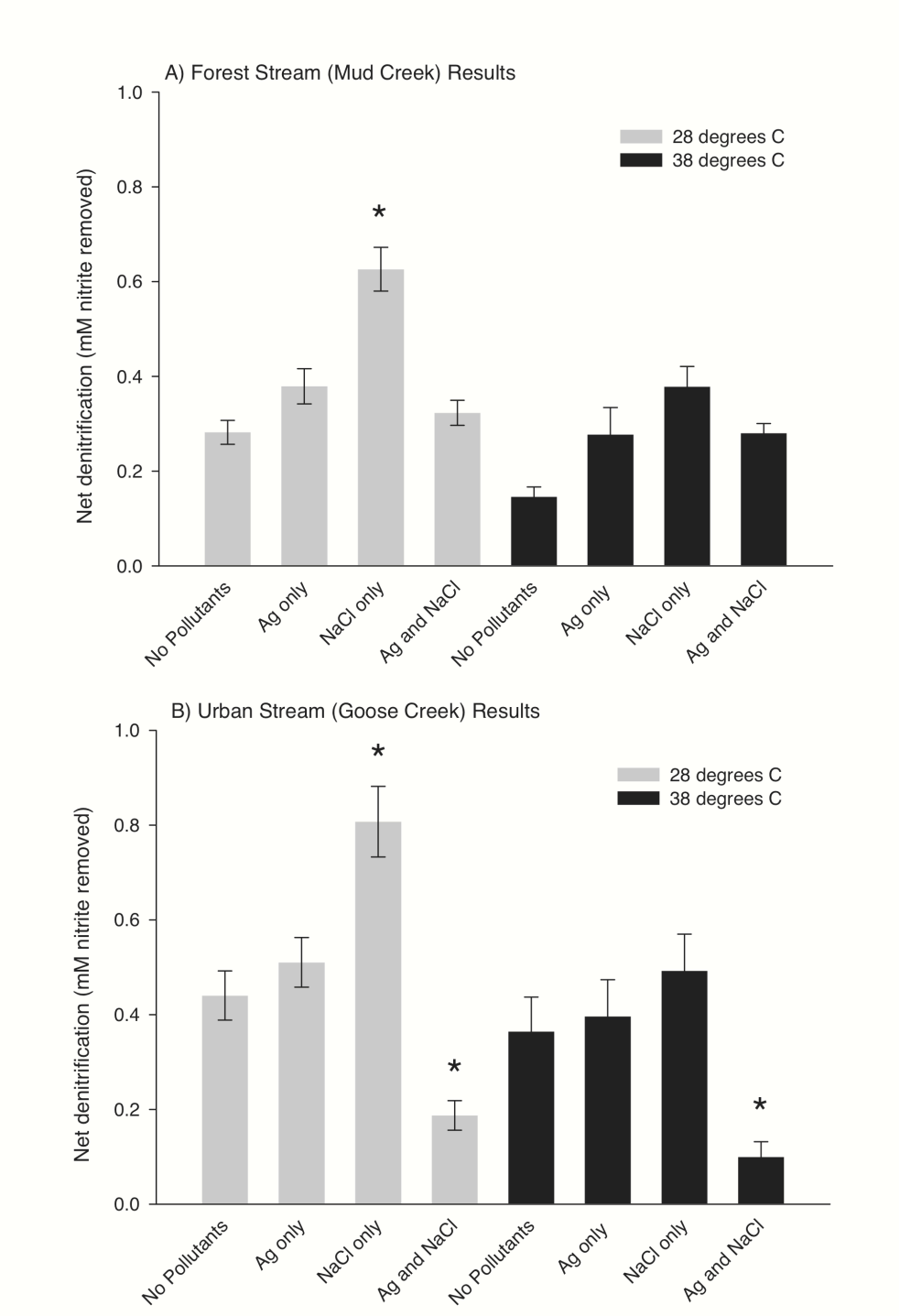

Figure 5: Net denitrification under different experimental conditions for A) forest and B) urban communities. Error bars represent ±1 standard error. Asterisks indicate a significant difference between reference and treated microcosms.

3.4 Discussion

The urban stream had higher chloride concentrations and streamwater temperatures than the forest stream. The legacy hypothesis therefore predicts greater resistance to the salt and temperature treatments for urban versus forest communities. This expectation was not met. Instead, net denitrification for both community types was stimulated (at 28°C) or unaffected (at 38°C) by the salt treatment. Net denitrification for both communities was similarly unresponsive to the temperature treatment. These results suggest that communities did not experience these treatments as stressful disturbances. The same could also be said for the silver treatment, which also did not lead to any changes in net denitrification for urban or forest communities.

The silver, salt, and temperature treatments on their own were moderate disturbances that did not negatively affect (and sometimes even stimulated) denitrifier communities. That is, these treatments had the same effect on urban and forest communities, regardless of any potential differences in initial community structure, possibly because both communities had multiple members that could continue to denitrify in the face of the moderate disturbance. The maintenance of high denitrification rates in the face of a more severe and selective disturbance, such as the *combined* silver and salt treatment, likely requires multiple tolerance mechanisms, such as efflux pumps, that are energy-intensive and costly (Gadd and Griffiths 1977) and potentially less widely distributed.

When subjected to the combined silver and salt treatment, urban communities experienced large, significant reductions in net denitrification. Forest communities, on the other hand, were highly resistant to the same treatment, despite a lack of ongoing exposure in the field to high silver and chloride concentrations. Silver concentrations were comparable in the urban and forest stream; both streams had concentrations below

the 2 ppm benchmark cited as potentially damaging to sediment biota (Jones, Suter, and Hull 1997). Chloride concentrations were significantly higher in the urban versus forest stream. The same pattern of resistance emerged when communities were exposed to the combined silver and salt treatment, together with the increased temperature treatment. Forest communities were unaffected, while urban communities suffered an even greater decrease in net denitrification (77% versus 58% decrease at 38 versus 28°C, respectively).

Since laboratory microcosms differed primarily in the type of inoculum given, initial community structure was likely an important factor in driving observed differences in functional resistance. Multiple aspects, including richness, evenness, and composition, define community structure. Based on the *nirK* TRFLP data, we can conclude with reasonable confidence that at least one subset of the urban and forest denitrifier communities had comparable compositions. While we cannot reliably estimate denitrifier diversity at the species level with TRFLP data, we can use TRFLP data to estimate diversity at a taxonomic resolution above the species level (i.e., the OTU level) (Blackwood et al. 2007; Fierer et al. 2007). Our results, therefore, suggest *nirK* denitrifier OTU diversity, in terms of both richness and evenness, was higher in the forest versus urban community.

According to the insurance hypothesis, greater diversity increases the odds of having community members that can respond favorably to a disturbance and/or functionally replacing important (but sensitive) members and, thereby, maintaining high functioning. Consistent with this theory, forest denitrifier communities were more diverse *and* more resistant than urban denitrifier communities. These findings correspond with that of a recent study documenting a positive relationship between denitrifier community evenness and resistance to salt stress (Wittebolle et al. 2009). Taken together, our results indicate that the insurance hypothesis provides a better

explanation of observed differences in functional resistance between urban and forest denitrifier communities than the legacy hypothesis.

Human impacts, such as land use change and climate change, often lead to increasingly compounded and severe disturbances (Paine, Tegner, and Johnson 1998). Urban streams, for instance, are extreme environments with multiple stressors, including not only pollution and high temperatures, but also flashy hydrology, altered channel stability, and increased nutrient concentrations (Walsh et al. 2005). As single stressors, pollution and increased temperature did not negatively affect community functionality. When combined, however, these stressors had a detrimental impact on the less diverse of the two experimental communities. These results suggest that microbial communities already under strong selection, such as those in urban streams, may be more sensitive to further environmental degradation, than potentially more diverse communities in relatively pristine environments.

4. Causes and functional consequences of denitrifying bacteria community structure in streams affected to varying degrees by watershed urbanization

4.1 Introduction

Urbanization is a particularly prominent type of land use change; more than 50 percent of the world's population currently resides in urban areas (UN-Habitat 2011). As low-lying points in landscapes, streams are disproportionately affected by the detrimental impacts of increasing urbanization, which include higher streamwater temperatures, increased nutrient and contaminant loading, and altered geomorphology and hydrology (reviewed by Walsh et al. 2005). This study examines the effects of watershed urbanization on the structure and function of denitrifying bacteria (denitrifier) communities in stream sediments.

Denitrifiers can transform bioavailable forms of nitrogen to inert, gaseous nitrogen (N_2) through a stepwise process known as denitrification (Groffman et al. 1999; Zumft 1997). Most denitrifiers are facultative anaerobes (Zumft 1997), using oxygen as the electron acceptor for organic carbon oxidation whenever available and nitrate as the next best alternative electron acceptor under hypoxic or anoxic conditions. Denitrification is one of the few ways to remove bioavailable nitrogen from water and can be considered a valuable ecosystem service because excessive bioavailable nitrogen contributes to serious water quality and human health problems (Sutton et al. 2011; Townsend et al. 2003; Vitousek et al. 1997). Denitrification is especially important in urban streams that receive large inputs of bioavailable nitrogen from sewer and wastewater effluents (Bernhardt et al. 2008).

The stepwise process of denitrification does not always go to completion and can sometimes produce nitrous oxide (N_2O), a powerful greenhouse gas that contributes to

climate change and ozone depletion. Factors thought to control the ratio of N_2O to N_2 produced by denitrification include not only abiotic factors like pH (Firestone, Firestone, and Tiedje 1980), but also biotic factors like the proportion of the denitrifier community with the gene for N_2O reductase (Philippot et al. 2011). Genomic analyses suggest that as much as one-third of denitrifiers capable of reducing nitrite lacks the gene for N_2O reductase, meaning that they can only produce N_2O as the end product of denitrification (Jones et al. 2008).

In initiating this study, we expected watershed urbanization to alter the structure (composition and diversity) of denitrifier communities through four key impacts on stream conditions: 1) higher temperatures, 2) greater substrate (nitrogen and organic carbon) availability, 3) increased contaminant concentrations (e.g., heavy metals), and 4) more frequent and severe scouring flows. While urbanization can sometimes also alter stream conditions in other ways (e.g., reduced baseflow), we focused in this study on those four impacts because they are the most consistent symptoms of what has been called the "urban stream syndrome" (Meyer, Paul, and Taulbee 2005; Paul and Meyer 2001; Walsh et al. 2005). The rationale for this hypothesis is based on previous findings of reduced richness and altered composition for algal, insect, and fish communities in streams draining urban watersheds (Walsh et al. 2005). Moreover, in the only published study on this topic to date, denitrifier communities in an urban versus non-urban stream had significantly different compositions (Perryman, Rees, and Walsh 2008).

We also expected urbanization to alter denitrification rates directly through changes to stream conditions and indirectly through microbial responses to altered stream conditions. For example, increased substrate supply might directly stimulate denitrification independently of changes to denitrifier community structure (Inwood, Tank, and Bernot 2007; Mulholland et al. 2008). At the same time, increased

contamination could indirectly alter denitrification by selecting for denitrifiers that could differ in either their efficiency of or proclivity for denitrification (Wallenstein et al. 2006; Zumft 1997). The rationale for this hypothesis is based in part on previous findings of decreasing nitrogen uptake in streams with increasing watershed urbanization (Inwood, Tank, and Bernot 2007; Meyer, Paul, and Taulbee 2005; Mulholland et al. 2008) and in part on growing evidence suggesting that microbial community structure can play a critical role in controlling ecosystem process rates (Allison and Martiny 2008; Bell et al. 2005; Cavigelli and Robertson 2000; Philippot and Hallin 2005).

To evaluate our general hypotheses of how watershed urbanization might affect denitrifier community structure and function, we characterized the composition and diversity of nitrite reducing and N_2O reducing denitrifier communities and measured denitrification potentials in 49 streams affected to varying degrees by watershed urbanization. Denitrification potential is the maximum denitrification rate achievable by the extant community (Groffman et al. 2006). Our specific research questions were: 1) How do temperature, substrate supply, contamination, and hydrology differ in their relative impacts on denitrifier community structure and denitrification potential? 2) What are the relative strengths of abiotic (e.g., temperature) versus biotic (composition and diversity) controls on denitrification potential? To address these questions, we developed a structural equation model (SEM) to statistically disentangle the multiple causal pathways involved in these complex ecosystems (Figure 6) (Grace 2006). Findings from this study provide an improved mechanistic understanding of how watershed urbanization influences the structure and function of ecologically important microbial communities.

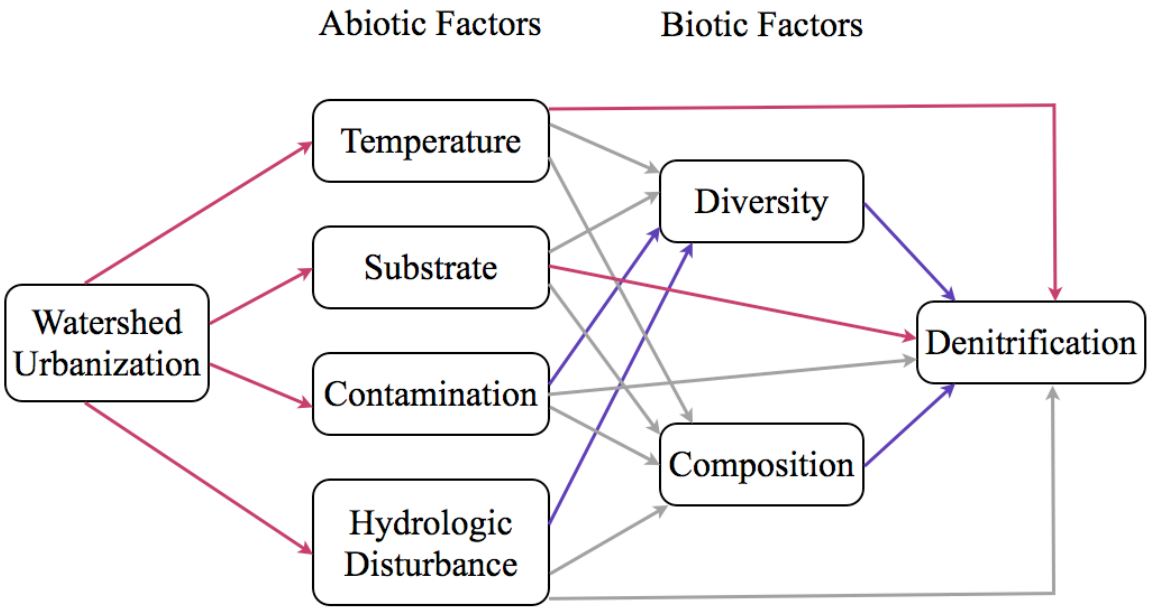

Figure 6: Path diagram illustrating hypothesized effects of watershed urbanization on denitrifier community structure and function (i.e., denitrification). Red paths indicate positive effects and blue paths indicate negative effects. Gray paths indicate no clear hypotheses for direction of effect.

4.2 Methods

4.2.1 Study streams

All study streams (n = 49) were located in the Triangle region of the North Carolina Piedmont (USA), a metropolitan area experiencing rapid population growth. In addition to heavily urbanized areas, such as Raleigh, Durham, and Chapel Hill, the region also contains large tracts of suburban development, agricultural land, and protected forest. Given the gradient of development intensities, the Triangle region provides a model landscape for studying the impacts of urbanization.

On May 19, 2009, we deployed HOBO® temperature loggers (Onset Computer Corporation, Bourne, MA, USA) in all study streams at once. Between May and August 2009, we re-visited each stream once to collect water and sediment samples, survey macroinvertebrate communities, and measure stream habitat metrics (e.g., width). We

collected sediments by randomly selecting five points within a 100 m study reach upstream of the temperature logger at each site. We then demarcated a 2 m segment of streambed centered on each selected point and used corers (6.35 cm diameter) to obtain multiple sediment cores until a total of at least 4,024 cm^3 of sediment was collected. Sediment cores were sieved (2 mm opening) and composited in the field and transported to the laboratory on ice. Subsamples for molecular analyses were stored at -80°C, while those for other analyses (e.g., heavy metals) were stored at 4°C.

This study focuses on the structure and function of stream denitrifier communities and their relationship to watershed and in-stream characteristics. Therefore this study focused only on a subset of variables we believed could strongly affect stream denitrifier community structure and function (Table 13). Companion papers focus on other aspects of the full dataset (see Somers et al. 2011 for study of streamwater temperatures).

Table 13: Watershed land use/land cover and environmental variables

Name	Description
Watershed urbanization	
% developed	Percent of watershed with developed land cover in 2005
traffic volume	Traffic volume, weighted by distance from stream
riparian buffer	Effective buffer area within 500 meters of stream
Streamwater temperatures	
min temperature	Mean daily minimum temperature
max temperature	Mean daily maximum temperature
mean temperature	Average of daily mean temperature
temperature range	Mean diel swing, calculated by subtracting daily minimum from the daily maximum and then averaging all daily ranges
max change	Maximum absolute value of temperature change between readings during a nine-day period of steady rain
Substrate supply	
NO_3-N	Streamwater nitrate concentration
NH_4-N	Streamwater ammonium concentration
TN	Streamwater total nitrogen concentration

Table 13 continued

Substrate supply continued	
TOC	Streamwater total organic carbon concentration
AFDM	Organic matter content of sediments, as ash-free dry mass
Contamination	
Ag	Sediment silver concentration, normalized by AFDM
Al	Sediment aluminum concentration, normalized by AFDM
As	Sediment arsenic concentration, normalized by AFDM
Cd	Sediment cadmium concentration, normalized by AFDM
Cr	Sediment chromium concentration, normalized by AFDM
Cu	Sediment copper concentration, normalized by AFDM
Ni	Sediment nickel concentration, normalized by AFDM
Pb	Sediment lead concentration, normalized by AFDM
Zn	Sediment zinc concentration, normalized by AFDM
Hydrology	
incision	Mean channel incision, calculated as channel depth at thalweg divided by bankfull width from 3 random cross sections
transitions	Number of flow habitat transitions per stream reach
storm surge	Maximum streamwater temperature change during 24-hour period coinciding with a storm event

4.2.2 Watershed urbanization

For each study stream, we used 2005 land use/land cover imagery (Sexton et al. *in review*) and GIS software (ArcGIS v.9.3, ESRI, Redlands, CA, USA) to calculate a number of indices representing different aspects of watershed urbanization intensity, such as percent developed cover, percent forest cover, road density, traffic volume, and streamside buffering (see Somers et al. *submitted* for the full set of indices and further details). For the purposes of this study, we chose three indices to represent the intensity of watershed urbanization: 1) percent developed land cover (% developed), 2) near stream traffic volume (traffic volume), and 3) effective riparian buffer (riparian buffer).

We calculated % developed as the cumulative watershed area classified as "low-density developed", "medium-density developed", or "high-density developed" (NLCD

classes 22, 23, and 24, respectively) divided by the total watershed area. We used % developed because the index is commonly used and intuitively simple to understand.

Traffic volume was calculated by computing the Euclidean distance from each point in the study stream to the nearest road. Based on the rationale that road impacts (e.g., heavy metals) decrease with increasing distance from the stream, we log-transformed calculated distances and weighted them by estimates of traffic volume in 2009 (NCDOT 2009).

Riparian buffer was an estimate of the effective naturally vegetated buffer area (forest) within 500 meters along hydrologic flowpaths to the stream. We included traffic volume and riparian buffer on top of % developed because they provide additional, non-overlapping information on the trajectory of urbanization in each watershed. Watersheds with comparable % developed values may have substantially different traffic volume and riparian buffer values, possibly as a result of distinct zoning configurations or restoration.

4.2.3 Streamwater temperatures

Temperature is known to play a key role in determining the types of bacteria that can persist in a particular environment and the rates at which they can grow (Bergey and Holt 1994). We used streamwater temperature data collected every 10 minutes over the research effort to calculate five metrics representing different aspects of temperature regimes (Somers et al. 2011). These included the minimum, maximum, and mean temperature, as well as the temperature range, all based on data recorded between May 19 and June 10, 2009. Minimum and maximum temperature were the mean daily minimum and maximum temperature, respectively. Mean temperature was the average of daily mean temperatures. Temperature range was the mean diel swing, calculated by

subtracting the daily minimum from the daily maximum and then averaging all the daily ranges.

We also calculated the maximum temperature change (max change) that occurred between readings during a nine-day period (May 24 to June 1, 2009) of steady rain during which there was less likely to be major disturbances from sediment burial (i.e., from violent storm disruptions) or drying up of the stream. Max change provides a measure of the degree of thermal stress stream inhabitants might experience from sudden, large changes in streamwater temperatures.

4.2.4 Substrate supply

Since denitrifers need nitrogen and carbon as substrates for denitrification, we focused on characterizing nitrogen and carbon chemistry in study streams. We measured nitrate (NO_3), ammonium (NH_4), total nitrogen (TN), and total organic carbon (TOC) concentrations on field filtered (glass microfiber, 0.7μm) streamwater samples collected on the same day as sediment samples that were used to characterize denitrifier communities and to measure denitrification potential.

All chemical analyses were done in duplicate. We measured NO_3-N concentrations with a Dionex ICS-2000 ion chromatograph with an AS-18 column (Dionex Corporation, Sunnyvale, CA, USA). NH_4-N was measured using the OPA method (Holmes et al. 1999) and analyzed on a fluorometer (Turner Designs, Sunnyvale, CA, USA). TN and TOC concentrations (as non-purgeable organic carbon) were measured on a Shimadzu TOC analyzer with a nitrogen module (Shimadzu Scientific Instruments, Columbia, MD, USA).

In addition to streamwater measurements described above, we also determined the organic carbon content of sediment samples by measuring ash-free dry mass (AFDM). AFDM is the amount of dry mass of a sediment sample that is organic and can,

therefore, be removed by combustion. To determine AFDM values, we weighed out five replicate 5 g sediment subsamples for each site, dried at 60°C, weighed to get dry mass, combusted at 400°C, and weighed again to get combusted mass. The difference between dry and combusted mass, divided by dry mass is AFDM.

4.2.5 Contamination

We expected contamination to have strong impacts on bacterial communities (Bååth 1989). While pharmaceuticals, herbicides, and other toxic chemicals can also contaminate urban streams (Kolpin et al. 2002), we chose to focus primarily on heavy metals, because 1) previous work has documented changes to denitrifier community structure and denitrification rates in response to heavy metal contamination (Magalhaes et al. 2007; Sakadevan, Zheng, and Bavor 1999; Throback et al. 2007) and 2) heavy metal concentrations are relatively easy to measure. We measured concentrations of nine heavy metals, including silver (Ag), aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn). Sources of heavy metals include runoff from roads and parking lots, as well as sewer, wastewater, and industrial effluents (Walsh et al. 2005).

To measure heavy metal concentrations, we re-sieved sediment subsamples (1 mm opening), dried them at 60°C for 48 hours, and then weighed out three 1 g subsamples per site for digestion (EPA method 3050B). Briefly, digestion involved adding 10 mL of 50% nitric acid, heated at 95°C, followed by another 5 mL of nitric acid (also heated to 95°C) to each sediment sample. Ten mL of 30% hydrogen peroxide were then added before diluting to 75 mL with double-deionized water and filtering samples (Whatman #41) (Whatman International Ltd., Kent, UK). Digested samples were analyzed for trace metals by inductively coupled plasma-mass spectrometry (Perkin-Elmer Elan 6000 ICP-MS, Perkin-Elmer, Waltham, MA, USA). For every 35 samples

analyzed, we also processed three replicates of certified reference materials STSD-3 (NRC, Institute for National Measurement Standards, Ottawa, Canada) and two method blanks.

To account for differences in organic carbon content among sediment samples, we normalized all heavy metal concentrations by AFDM (Liu et al. 2003; Loring 1991). Normalized values were then averaged across the three analytical replicates processed for each site. All heavy metal data are therefore reported as mean $\mu g\ g^{-1}$ AFDM sediment, except for Al, which is reported as mean $mg\ g^{-1}$ AFDM sediment.

4.2.6 Hydrology

We used two in-stream habitat metrics, mean bank incision and mean number of habitat transitions, as proxies to describe stream hydrology. Our primary interest was in capturing the degree of hydrologic disturbance denitrifiers experience in our study streams. Bank incision is typically greater in streams that regularly encounter sudden, intense, and sustained peaks in stream flow following rain events (Hardison et al. 2009). Increased hydrologic disturbance may also be associated with fewer habitat transitions as violent storm peaks are likely to homogenize the physical structure of stream reaches by dislodging debris and other structures (Violin et al. 2011).

Habitat metrics were measured along 100 m reaches upstream of where temperature loggers were deployed. We conducted cross sections at three randomly chosen points along each reach. Cross sections involved using a measuring tape (stretched across the top of opposite banks) to determine bank-to-bank width and then recording the height from the tape to at least seven points: top of one bank, bottom of one bank, one water edge, thalweg, other water edge, bottom of other bank, and top of other bank. Mean bank incision was calculated as channel depth (bank height to streambed) at thalweg, divided by bankfull width, averaged over the three cross

sections. At each reach, we also counted the total number of habitat transitions between pool, riffle, and run.

We viewed the maximum difference between temperature readings during a 24-hour period coinciding with a large storm event (storm surge) to be a consequence of (and thus, a proxy) for indirectly characterizing hydrologic disturbance. Streamwater temperatures can be thought of a tracer for how much and how quickly runoff from watersheds is routed into streams (Herb et al. 2008). Highly developed watersheds with a lot of hot impervious cover (e.g., dark parking lots) and little permeable land will deliver large quantities of warm water very quickly into receiving streams during summer storms (Gall and Dubose 1990). Streams with flashy hydrology are therefore likely to experience large heat pulses (i.e., high storm surge values) following summer storms. The exception would be streams in urban watersheds with impervious cover that does not heat up easily, perhaps due to green building practices, such as green roofs. We do not expect this to be the case for most of the urban landscapes in this study, but this potential exception highlights the fact that these are approximate and imperfect measures of hydrologic disturbance.

4.2.7 Denitrifier community structure

We used terminal restriction fragment length polymorphism (TRFLP), a cultivation-independent, molecular fingerprinting method, to characterize denitrifier communities in sediment samples. While the TRFLP method does not provide reliable estimates of species richness, it is highly cost-effective and generally accepted as an appropriate and efficient means of assessing operational taxonomic unit (OTU) richness and overall microbial community composition among samples (Fierer et al. 2007; Osborn, Moore, and Timmis 2000).

Since the focus of this study was on assessing variability in communities among sites, rather than within each site, we extracted DNA from sediment subsamples taken from field composited sediment cores. Duplicate extractions were done per site with PowerSoil kits (MoBio Laboratories, Carlsbad, CA, USA), according to manufacturer instructions. Following extraction, we performed polymerase chain reactions (PCRs) with Hot Start Taq Master Mix (Sigma-Aldrich, St. Louis, MO, USA) and one of two different primer sets: 1) a nirK primer set for the copper containing form of nitrite reductase and 2) a nosZ primer set for N_2O reductase. An estimated one-third of denitrifiers capable of reducing nitrite do not have the capacity to reduce N_2O (Jones et al. 2008); N_2O reducing denitrifiers are also generally less abundant than nitrite reducing denitrifiers (Philippot et al. 2011). The two primer sets therefore provide different perspectives of denitrifier communities at each site.

The *nirK* primer set was nirK1F (5′- GG(A/C)ATGGT(G/T)CC(C/G)TGGCA, FAM labeled) and nirK5R (5′-GCCTCGATCAG(A/G)TT(A/G)TGG) (Braker, Fesefeldt, and Witzel 1998). For *nirK* PCRs, each of 33 cycles consisted of 30s at 95°C, 30s at 46°C, and 45s at 72°C. The *nosZ* primer set was nosZ-F (5′- CG(C/T)TGTTC(A/C)TCGACA GCCAG, FAM labeled) and nosZ1622R (5′- CGC(G/A)A(C/G)GGCAA(G/C)AAGGT (G/C)CG) (Throback et al. 2004). For *nosZ* PCRs, each of 35 cycles consisted of 30s at 94°C, 60s at 53°C, and 60s at 72°C.

PCR products were cleaned with Qiaquick PCR purification kits (Qiagen, Germantown, MD, USA), checked for appropriate sizes by agarose gel electrophoresis, and then digested with endonuclease HaeIII for *nirK* products and endonuclease MnlI for *nosZ* products to generate TRFLP profiles. All endonucleases were from New England Biolabs, Ipswich, MA, USA. Subsequent electrophoresis runs were done on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

To process TRFLP data, we used T-REX software (Culman et al. 2009) to define a baseline fluorescence threshold for filtering true peaks from background noise and to align terminal restriction fragments (TRF). The filtering algorithm in T-REX discards peaks that do not meet a user-specified standard deviation limit (in this case, we chose to use one standard deviation) (Abdo et al. 2006). After filtering, we aligned TRFs using a clustering threshold of 0.5 base pair (Smith et al. 2005) and eliminated TRFs that appeared in less than five percent of samples in the dataset. Each unique TRF represents an OTU. For subsequent analyses of compositional differences between sites, we transformed the processed TRFLP data into a matrix of relativized (by site-wise total relative fluorescence units) abundances.

We calculated two community diversity metrics: OTU richness and evenness. OTU richness is the total number of OTUs in each TRFLP replicate, averaged across TRFLP replicates. OTU evenness was quantified by calculating the Gini coefficient (G) for each TRFLP replicate, then averaging across replicates. G ranges from zero to one. G values of zero represent perfectly even communities, while values close to one represent highly uneven communities dominated by few taxa (Naeem 2009; Wittebolle et al. 2009).

4.2.8 Denitrification potential

To quantify denitrification potential, we conducted denitrification enzyme activity (DEA) assays, which are short laboratory incubations conducted at room temperature under optimal conditions (i.e., anoxia and unlimited substrate availability) (Tiedje 1994). For each site, we prepared five replicate incubation slurries by weighing 10g of sediment into Erlenmeyer flasks (125 mL) and adding 20 mL of stock media solution (0.72 g potassium nitrate, 0.5 g glucose, and 0.125 g chloramphenicol in 1 L of double de-ionized water). Flasks were made airtight with butyl rubber stoppers (Grace, Deerfield, IL, USA) and evacuated and flushed with nitrogen gas (N_2) for three

successive cycles to achieve anaerobic conditions. We started incubations by adding 10 mL of acetylene, which inhibits N_2O reduction and allows N_2O to accumulate in the flask headspace during the 90 minute incubation. We withdrew gas samples at the start of the incubation and every 30 minutes after. Chloramphenicol inhibits the synthesis of new enzymes and bacterial communities are unlikely to change significantly during the short incubation period (Bernot et al. 2003).

The potential rates measured using DEA assays are estimates of the maximum denitrification rates achievable by the extant community under optimal conditions during short incubations that do not allow enough time for shifts in denitrifier community structure arising from growth or syntheses of new denitrification enzymes. Potential denitrification rates are, therefore, a function of: 1) the concentration of denitrification enzymes, which reflects stream conditions at time of sampling and 2) denitrifier community structure, which reflects a legacy of stream conditions leading up to time of sampling. DEA assays are widely accepted as a valid means of comparing denitrification rates among sites (Groffman et al. 2006).

N_2O concentrations were measured by using a Teledyne Tekmar 7000 headspace autosampler (Teledyne Tekmar, Mason, OH, USA) to inject samples into a Shimadzu GC-17A ver.3 gas chromatograph with a Porapak Q column and electron capture detector (injector temperature = 380°C, column temperature = 80°C, detector temperature = 340°C, with N_2 carrier gas). Bunsen coefficients were used to calculate N_2O concentrations and final estimates of denitrification potential were calculated as the mean N_2O production rate observed over 30 minute intervals, divided by the dry mass of sediments (ng N g sediment^{-1} hr^{-1}).

4.2.9 Exploratory analyses of denitrifier community structure

To visualize patterns of dissimilarity in denitrifier community composition among study streams, we conducted non-metric multidimensional scaling (NMS) ordinations with the ecodist package (Goslee and Urban 2007) in R (R 2.3.1., 2006, distributed freely at http://www.R-project.org/). NMS ordinations were done using relativized TRFLP data, Bray-Curtis distance measures, a random starting configuration, and 500 runs with real data (Rees et al. 2004). NMS ordinations map samples into a reduced ordination space that preserves the rank order of ecological distances among samples (McCune and Grace 2002). Each sample is then associated with ordination axes scores that can be used to represent different components of variation in denitrifier community composition among samples.

To compare denitrifier profiles based on *nirK* versus *nosZ* primers, we conducted a simple Mantel test (Mantel 1967) with Bray-Curtis dissimilarity matrices using the ecodist package (Goslee and Urban 2007) in R. We used Monte Carlo randomization tests (9999 permutations) to calculate *p*-values. The standardized Mantel statistic (*r*), which ranges from -1 to 1, provides a measure of the strength of the relationship between matrices. Zero indicates no relationship while values close to -1 or 1 indicate a strong relationship (McCune and Grace 2002). A large and significant *r* would suggest that the distribution of denitrifiers with the copper containing form of nitrite reductase (*nirK*) is highly correlated with the distribution of denitrifiers with N_2O reductase (*nosZ*).

To determine which of the environmental variables listed in Table 13 were most closely associated with denitrifier community composition, we fitted vectors onto the NMS ordinations using the envfit function in vegan (Oksanen et al. 2010) in R. The significance of each fitted vector was assessed by 9999 random permutations of the

variable on all axes conjointly. If a variable is highly correlated with one axis, but not at all correlated with the other axis, the overall association will likely not be significant.

We also conducted partial Mantel tests to explore the relative impacts of temperature, nutrients, contamination, and hydrology on denitrifier community composition. We used the ecodist package (Goslee and Urban 2007) in R and 9999 Monte Carlo randomizations for each test. We ran one partial Mantel test for each denitrifier community type (i.e., *nirK* or *nosZ*) and urban impact category (i.e., temperature, substrate supply, contamination, and hydrology). For example, to assess the influence of temperature on *nirK* communities, we conducted a partial Mantel test comparing a matrix of *nirK* Bray-Curtis dissimilarities against temperature, given substrate supply, contamination, and hydrology matrices. Environmental matrices were based on Mahalanobis distance measures and the environmental variables listed in Table 13. The hydrology matrix, for instance, included incision, transitions, and storm surge. Due to missing values, the dataset was reduced to 37 sites for partial Mantel tests.

To determine whether OTU richness and evenness differed among watershed land use types (urban, forest, and agriculture), we conducted one-way analysis of variance (ANOVA) with fixed factors in R. Land use type classifications were based on whatever land cover had the highest percentage in the watershed. For watersheds that had similar percentages for two land cover types, we classified sites as urban if the urban land cover exceeded 35%. We also conducted bivariate regressions of OTU richness and evenness against the environmental variables in Table 13.

To explore whether denitrifier community composition differed among watershed land use types, we used the adonis function in the vegan package (Oksanen et al. 2010) in R to conduct permutational multivariate analysis of variance (perMANOVA) (Anderson 2001). perMANOVA is similar to redundancy analysis

(Legendre and Anderson 1999) and calculates a pseudo *F*-statistic by comparing the total variance explained by sample identity (in this case, urban, forest, or agriculture) to that explained by random permutations (n = 999) of sample identities. We used relativized abundances and Bray-Curtis distance measures.

4.2.10 Exploratory analyses of denitrification potential

To test whether denitrification potentials differed among watershed land use types (urban, forest, and agriculture), we conducted a one-way ANOVA with fixed factors in R. We $\log_{10}$-transformed denitrification potentials to improve normality prior to the ANOVA. We also examined linear regressions of log-denitrification against watershed land use/land cover, nitrogen, and carbon variables. Nitrogen, carbon, traffic volume, and riparian buffer values were $\log_{10}$ transformed, while % developed was arcsine square root transformed prior to analyses.

4.2.11 Structural equation modeling (SEM)

SEM is designed to allow researchers to specify and simultaneously evaluate multiple hypothesized causal pathways, which is especially valuable for understanding complex environmental systems that are difficult to analyze with univariate models. SEM results include estimates of the partial strength of each pathway (pathway coefficients) and standardized coefficients permit comparisons in common units. An assessment of the overall model-data fit is based on a comparison of the covariance structure implied by the model versus the covariance structure of the actual data. Overall model fit is particularly sensitive to missing pathways (Grace 2006, 2008). While we feel the general theoretical relationships providing the context for modeling are well supported (Figure 6), *a priori* knowledge of the precise form of relationships and of which candidate measures for a concept would be most informative/predictive was

lacking. For this reason, our application of SEM can best be described as "model building" (Grace 2006).

We began the modeling process by first building a series of initial SEMs based on our *a priori* hypotheses relating urban impacts to denitrifier community structure and function (Figure 6). To maximize interpretability and strive for model simplicity, we sought to select one observed variable to represent each conceptual variable (Grace et al. 2010). Our choices of which observed variable to use to represent each impact were informed by strength of association and conceptual justification.

We simplified the model by sequentially removing the least significant path until all paths left were at least marginally significant ($p \leq 0.10$) (Chaudhary et al. 2009). Models created in this somewhat liberal fashion serve as a provisional representation of the study system and require future studies to more rigorously test the generality of pathways included in the model (e.g., Larson and Grace 2004). All SEM analyses were done using maximum likelihood procedures and the AMOS software (v16.0, IBM, SPSS, Armonk, New York, USA) (Grace and Pugesek 1998).

4.3 Results

4.3.1 Study stream characteristics

The 49 study streams encompassed a wide variety of watershed land use/land cover characteristics (Supplementary Materials, Figure 10). For instance, watershed % developed ranged from 0 to 99, with a mean of 27%. Study streams also had varying thermal regimes, with the mean temperature recorded over the research effort ranging from 18 to 23 and averaging 21°C (Supplementary Materials, Figure 11). Substrate supply varied widely among study streams, with NO_3-N concentrations ranging between $5x10^{-4}$ and 2.34 and averaging 0.43 mg L^{-1} (Supplementary Materials, Figure 12). For reference, the permissible level for NO_3-N in drinking water in the US is 10 mg L^{-1}

(USEPA 1987). For protecting sensitive freshwater invertebrates, fish, and amphibians, a maximum level of 2.0 mg L^{-1} NO_3-N has been recommended (Camargo, Alonso, and Salamanca 2005). Study streams also encompassed a range of heavy metal concentrations (Supplementary Materials, Figure 13). Lead concentrations, for instance, ranged from 1.25 to 44.52 and averaged 7.99 μg g^{-1} AFDM sediment. Finally, the study streams also varied widely in terms of hydrology, with mean incision ranging from 0.04 to 0.44 and averaging 0.22 m (Supplementary Materials, Figure 14).

Study streams with high watershed % developed values tended to also have high traffic volume and low riparian buffer values (Table 14). % developed and traffic volume was positively correlated with many of the temperature, substrate, contamination, and hydrologic variables. Riparian buffer was negatively correlated with many of the temperature variables, two substrate variables, and one hydrology variable.

Table 14: Pearson's product-moment correlation coefficients between watershed land use/land cover and environmental variables

Name	arc sqrt-% developed	traffic volume	riparian buffer
arc sqrt-% developed			
traffic volume	**0.617** $^{<0.001}$		
riparian buffer	**-0.297** $^{0.038}$	-0.213	
min temperature	**0.362** $^{0.017}$	0.232	**-0.352** $^{0.021}$
max temperature	**0.320** $^{0.036}$	**0.269** $^{0.081}$	**-0.415** $^{0.006}$
mean temperature	**0.311** $^{0.042}$	0.216	**-0.388** $^{0.010}$
temperature range	0.091	0.165	**-0.262** $^{0.090}$
Log-max change	**0.715** $^{<0.001}$	**0.719** $^{<0.001}$	**-0.255** $^{0.099}$
Log-NO_3	0.213	-0.241	-0.234
Log-NH_4	**0.265** $^{0.072}$	0.051	-0.013
Log-TN	**0.417** $^{0.004}$	**0.381** $^{0.008}$	**-0.335** $^{0.021}$
Log-TOC	0.084	0.227	0.016
Log-AFDM	**-0.357** $^{0.013}$	-0.006	**-0.251** $^{0.085}$
Log-Ag	0.157	-0.146	0.188
Log-Al	**0.525** $^{<0.001}$	0.144	-0.122
Log-As	0.015	0.089	0.043
Log-Cd	**0.770** $^{<0.001}$	**0.445** $^{0.002}$	-0.153
Log-Cr	0.180	-0.017	-0.012

Table 14 continued

Name	arc sqrt-% developed	traffic volume	riparian buffer
Log-Cu	0.139	-0.024	-0.055
Log-Ni	**0.697** $^{<0.001}$	**0.435** $^{0.002}$	-0.198
Log-Pb	**0.727** $^{<0.001}$	**0.320** $^{0.027}$	-0.172
Log-Zn	**0.584** $^{<0.001}$	**0.312** $^{0.030}$	-0.222
incision	0.177	**0.248** $^{0.093}$	0.020
transitions	0.148	-0.011	0.031
Log-storm surge	**0.635** $^{<0.001}$	**0.436** $^{0.003}$	**-0.279** $^{0.070}$

Notes: Coefficients in bold have $p \leq 0.10$ (exact values in superscripts).

4.3.2 Denitrifier community structure

The convergent, final three-dimensional solution for the *nirK* ordination had a stress value of 21.35 (Figure 7). R^2 for the first, second, and third axes were 0.38, 0.22, and 0.16, respectively (total $R^2 = 0.77$). The only environmental variable that was significantly correlated with the *nirK* ordination was thermal stress (max change) (Table 15).

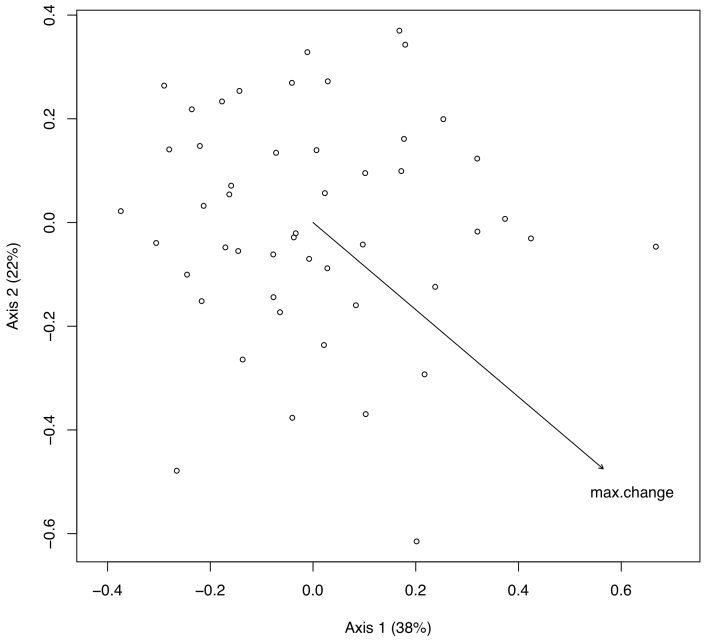

Figure 7: First two axes of three-dimensional NMS ordination of *nirK* communities. Final stress was 21.35 and total R^2 was 0.77. Arrows represent fitted vectors with $p \leq 0.05$.

Table 15: Fitted vectors on the NMS ordination of *nirK* denitrifier communities

	Axis 1	Axis 2	Axis 3	R^2	*p*
temp range	0.965	-0.031	0.260	0.186	0.070
max change	0.646	-0.596	-0.476	0.294	0.001*

Notes: * $p \leq 0.05$. Only showing details for fitted vectors with $p \leq 0.10$.

The convergent, final three-dimensional solution for the *nosZ* ordination had a stress value of 17.50 (Figure 8). R^2 for the first, second, and third axes were 0.47, 0.31, and 0.09, respectively (total $R^2 = 0.87$). NH_4, AFDM, Cd, Pb, and Zn were significantly correlated with the *nosZ* ordination (Table 16). See Appendix C for tables of raw data.

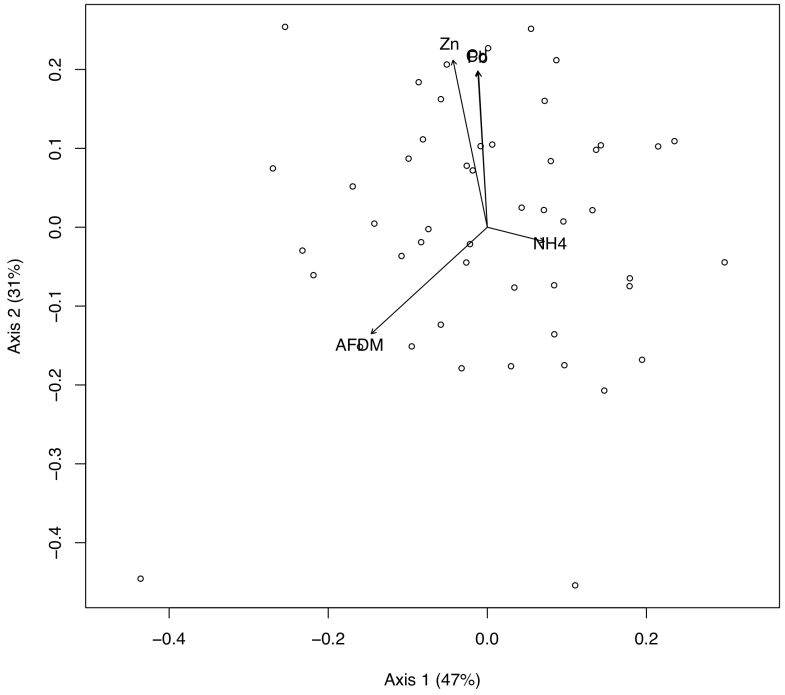

Figure 8: First two axes of the three-dimensional NMS ordination of *nosZ* communities. Final stress was 17.50 and total R^2 for was 0.87. Arrows represent fitted vectors with $p \leq 0.05$. Pb and Cd arrows are on top of each other.

Table 16: Fitted vectors on the NMS ordination of *nosZ* denitrifier communities

	Axis 1	Axis 2	Axis 3	R^2	*p*
max change	0.029	0.837	0.546	0.195	0.061
NH_4	0.305	-0.083	-0.949	0.409	0.003*
AFDM	-0.713	-0.695	0.092	0.271	0.019*
Al	0.127	0.968	-0.216	0.171	0.093
As	-0.905	0.186	-0.383	0.165	0.087
Cd	-0.056	0.971	0.233	0.295	0.013*
Ni	-0.079	0.997	-0.012	0.210	0.050*
Pb	-0.041	0.997	-0.070	0.283	0.023*
Zn	-0.294	0.955	0.047	0.355	0.002*
storm surge	0.291	0.757	0.586	0.176	0.088

Notes: * $p \leq 0.05$. Only showing details for fitted vectors with $p \leq 0.10$.

nirK and *nosZ* communities were not significantly correlated (simple Mantel test: $r = -0.058$, $p = 0.619$). Partial Mantel tests comparing *nirK* and environmental matrices found no significant associations with temperature ($r = -0.152$, $p = 0.204$), substrate supply ($r = -0.093$, $p = 0.464$), contamination ($r = -0.006$, $p = 0.971$), or hydrology ($r = 0.031$, $p = 0.787$). Partial Mantel tests comparing *nosZ* and environmental matrices indicated a significant association with substrate supply ($r = 0.324$, $p = 0.049$). Correlations with the other three urban impact categories were not significant (temperature: $r = 0.106$, $p = 0.363$; contaminants: $r = -0.065$, $p = 0.669$; hydrology: $r = 0.001$, $p = 0.996$).

nirK richness ranged from 5 to 137, with a mean of 71.02 and standard deviation of 29.29 OTUs. *nosZ* richness ranged between 12 and 166, with a mean of 96.67 and a standard deviation of 32.66 OTUs. *nirK* evenness (G) ranged between 0.68 and 0.98 and had a mean of 0.83 and a standard deviation of 0.07. *nosZ* evenness (G) ranged from 0.87 to 0.99, with a mean of 0.93 and a standard deviation of 0.03.

ANOVA results suggested no significant differences in denitrifier richness among watershed land use types (*nirK*: $F_{2,46} = 0.153$, $p = 0.858$ and *nosZ*: $F_{2,46} = 0.828$, $p = 0.443$). The same was also true for denitrifier evenness (*nirK*: $F_{2,46} = 1.053$, $p = 0.357$ and *nosZ*: $F_{2,46} = 0.880$, $p = 0.422$). Pairwise correlations only revealed one significant correlation between denitrifier diversity and environmental variables – that between *nirK* richness and thermal stress (max change) (Table 17).

Table 17: Pearson's product-moment correlation coefficients with denitrifier diversity

	nirK OTU		*nosZ* OTU	
	Richness	Evenness (G)	Richness	Evenness (G)
min temp	0.247	-0.115	-0.155	0.127
max temp	0.127	0.036	-0.130	0.064
mean temp	0.228	-0.075	-0.122	0.075
temp range	-0.090	0.201	-0.027	0.049
Log-max change	**-0.345** $^{0.023}$	**0.300** $^{0.051}$	-0.083	0.128
Log-NO_3	0.086	-0.021	-0.080	0.084
Log-NH_4	-0.005	0.024	-0.116	0.083
Log-TN	0.139	-0.094	0.015	-0.073
Log-TOC	0.110	-0.124	0.018	-0.130
Log-AFDM	-0.044	0.071	0.058	-0.131
Log-Ag	0.018	-0.005	-0.091	0.213
Log-Al	-0.012	-0.036	-0.053	0.129
Log-As	0.026	-0.134	-0.076	0.074
Log-Cd	0.123	-0.209	-0.090	0.080
Log-Cr	0.015	-0.058	0.067	0.021
Log-Cu	0.025	-0.020	0.132	-0.131
Log-Ni	-0.003	-0.031	0.012	0.013
Log-Pb	0.070	-0.091	-0.059	0.078
Log-Zn	0.062	-0.116	0.096	-0.105
incision	-0.108	0.129	0.052	-0.112
transitions	-0.040	-0.064	-0.157	0.086

Table 17 continued

	nirK OTU		*nosZ* OTU	
	Richness	Evenness (G)	Richness	Evenness (G)
Log-storm surge	-0.232	0.251	-0.224	0.209

Notes: To improve normality, some variables were log_{10}-transformed. Pearson product-moment correlation coefficients in bold have $p \leq 0.10$ (exact values given in superscripts). Positive correlations with richness indicate increasing richness with increases in the environmental variable (e.g., min temp). Positive correlations with G values (evenness) indicated decreasing evenness with increases in the environmental variable.

There were no significant differences in *nirK* community composition among watershed land use types (perMANOVA: $F_{2,46} = 1.101$, $p = 0.260$). *nosZ* community composition was marginally significantly different among watershed land use types (perMANOVA: $F_{2,26} = 1.559$, $p = 0.097$).

4.3.3 Denitrification potential

There were no significant differences in log-denitrification potential among watershed land use types (ANOVA: $F_{2,46} = 0.513$, $p = 0.602$). Mean values for urban, forest, and agricultural streams were 139.26 (standard deviation = 2.65, n = 16 sites), 142.45 (standard deviation = 2.25, n = 28 sites), and 93.29 (standard deviation = 2.38, n = 5 sites) ng N g sediment^{-1} hr^{-1}. Overall, denitrification potentials ranged from 18.73 to 940.80, with a mean of 135.42 and a standard deviation of 2.37 ng N g sediment^{-1} hr^{-1}.

Linear regressions of log-denitrification potential against log-NO_3, log-NH_4, log-TOC, and arcsine square root-% developed were not significant (Table 18). Linear regressions of log-denitrification against log-TN, log-AFDM, and log-traffic volume were significant and positive. The linear regression of log-denitrification against log-riparian buffer was significant and negative.

Table 18: Linear regressions of denitrification potential against watershed land use/land cover and nitrogen and carbon concentrations

	F-ratio	R^2	*p*	intercept	slope
% developed	$F_{1,47} = 0.48$	0.010	0.490	2.09	0.09
traffic volume	$F_{1,47} = 5.84$	0.111	0.020*	1.86	0.08
riparian buffer	$F_{1,47} = 6.86$	0.127	0.012*	2.67	-0.32
NO_3	$F_{1,45} = 1.79$	0.038	0.188	2.22	0.12
NH_4	$F_{1,45} = 3.89$	0.080	0.055	2.43	0.15
TN	$F_{1,45} = 10.90$	0.195	0.002*	2.22	0.53
TOC	$F_{1,45} = 0.07$	0.002	0.789	2.10	0.07
AFDM	$F_{1,46} = 10.14$	0.181	0.003*	2.13	0.52

Notes: * $p \leq 0.05$. All variables except % developed were $\log_{10}$-transformed. % developed was arcsine square root transformed. Degrees of freedom vary because of missing values for some sites.

4.3.4 Structural equation modeling (SEM) results

In the final SE model, we chose to represent the following concepts with the stated indicators: the temperature concept with temperature range, substrate supply with TN, contamination with lead concentrations, and hydrology with storm surge (based on information presented in Supplementary Materials, Tables 20 to 23).

Temperature range was the most highly correlated with denitrification (Supplementary Materials, Table 20) and also associated with denitrifier composition (Table 15). TN was the most highly correlated with denitrification and also correlated with one other measure of substrate supply (Supplementary Materials, Table 21). Lead was correlated with six other heavy metals (Supplementary Materials, Table 22) and denitrifier composition (Table 16). Although aluminum was similarly related to other heavy metals and denitrifier composition, it was not as highly correlated with watershed land use/land cover variables as lead (Table 14). Finally, storm surge was the most

highly correlated with denitrification (Supplementary Materials, Table 23) and also associated with denitrifier composition (Table 16).

Denitrifier community structure was represented by *nirK* ordination axis 2, *nirK* evenness (G), *nosZ* ordination axis 2, and *nosZ* evenness (G) in the SEM. Although other *nirK* and *nosZ* ordination axes appeared to be more highly correlated with denitrification potential (Supplementary Materials, Table 24), these univariate relationships were not informative in the context of the SEM and were, therefore, excluded. Moreover, because the composition and diversity metrics chosen for each denitrifier group were highly correlated, we allowed their errors to correlate in the model. This error correlation was deemed theoretically justified because the composition and diversity metrics for each group were based on amplified gene products generated using the same primers and DNA extractions.

Final model results were found to be stable and the fit between model expectations and data acceptable ($\chi^2 = 34.60$, df = 25, $p = 0.096$). After removing non-significant pathways, model results were still stable and the fit between model expectations and data became quite close ($\chi^2 = 29.79$, df = 36, $p = 0.758$; Figure 9 and Table 19). The simplified model explained 63% of the variation in denitrification potential among streams. Significant paths ($p \leq 0.05$) to denitrification potential included those from temperature range, TN, and *nirK* axis 2. Marginally significant ($p \leq 0.10$) paths to denitrification potential included those from *nosZ* axis 2 and *nosZ* evenness. The only significant path to *nirK* axis 2 was from TN ($p = 0.011$). Significant paths to *nosZ* axis 2 included those from TN ($p = 0.006$) and Pb ($p < 0.001$). Marginally significant paths to *nosZ* evenness included those from TN ($p = 0.096$) and storm surge ($p = 0.088$).

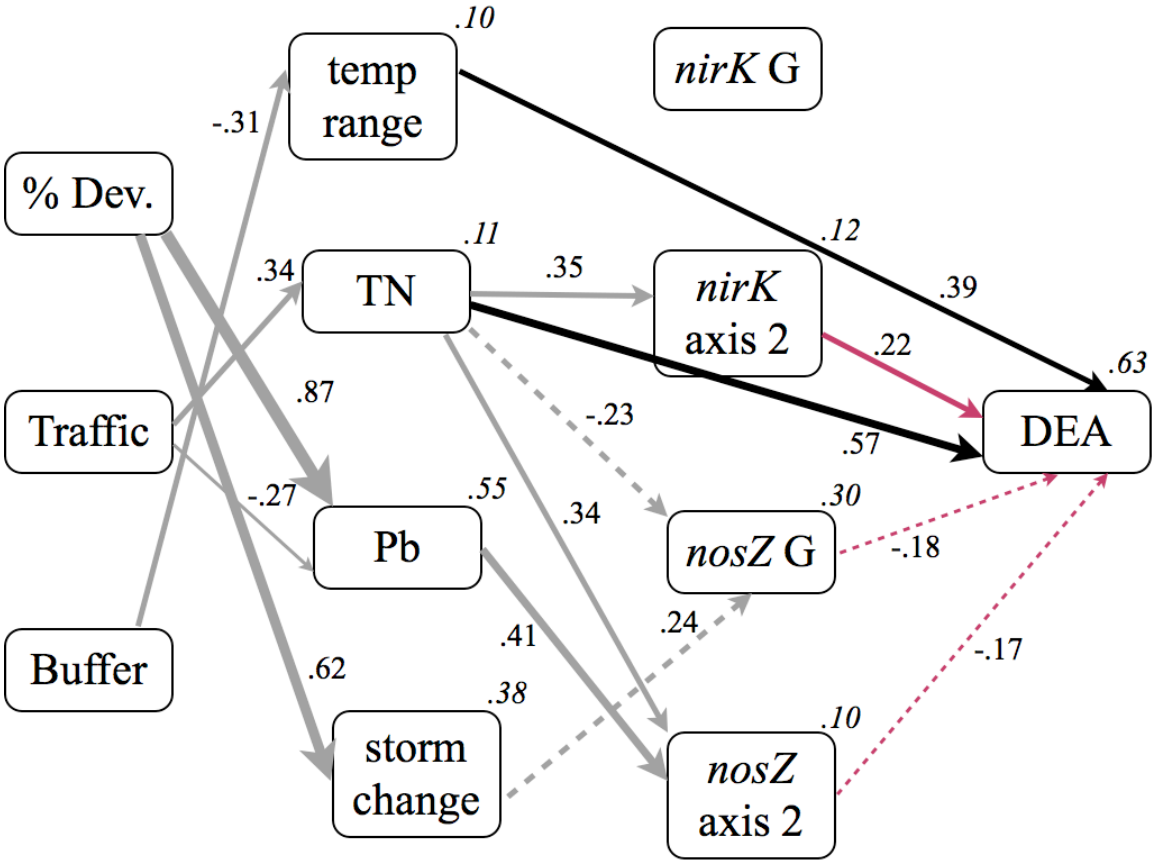

Figure 9: The final, simplified SEM of the direct (black) and indirect (red) pathways through which urban impacts associated with watershed urbanization affect denitrification. Values associated with arrows are the standardized path coefficients. Arrow thickness is roughly proportional to estimated strength of the relationship. Solid arrows have $p \leq 0.05$ and dashed arrows have $p \leq 0.10$. Values on the top right corner of boxes indicate the proportion of variation explained for that variable.

Table 19: Standardized direct and total effects of in-stream environmental variables

Variable	Denitrification		*nirK* axis 2		*nosZ* G		*nosZ* axis 2	
	Direct	Total	Direct	Total	Direct	Total	Direct	Total
temp range	0.385	0.385	0.000	0.000	0.000	0.000	0.000	0.000
TN	0.574	0.636	0.346	0.346	-0.233	-0.233	0.339	0.339
Pb	0.000	-0.068	0.000	0.000	0.000	0.000	0.407	0.407
storm surge	0.000	-0.043	0.000	0.000	0.235	0.235	0.000	0.000
nirK axis 2	0.221	0.221						
nosZ G	-0.183	-0.183						
nosZ axis 2	-0.168	-0.168						

4.4 Discussion

Watershed urbanization is known to cause many changes in receiving streams, a phenomenon termed the "urban stream syndrome" (Walsh et al. 2005). Findings from this study indicate that both the composition and diversity of denitrifier communities are affected by the urban stream syndrome. Moreover, our structural equation model of abiotic and biotic drivers of denitrification was consistent with the data and captured 63% of the variation in denitrification potential among streams investigated in this study. These findings suggest that recent observations of decreasing denitrification efficiency with increasing urbanization (Inwood, Tank, and Bernot 2007; Meyer, Paul, and Taulbee 2005; Mulholland et al. 2008) may be explained in part by microbial responses to urban impacts.

4.4.1 How do urban impacts affect denitrifier community structure?

Of the four classes of urban impacts (temperature, substrate supply, contamination, and hydrology) we identified as potentially important drivers of denitrifier community structure, we found that *nirK* composition responded to temperature, while *nosZ* composition responded to substrate supply and contamination. There was no evidence of a relationship between either *nirK* or *nosZ* composition and hydrology, although this may simply reflect our imperfect measures of hydrology.

As for the relative strength of impacts, no single impact stood out as the strongest driver of *nirK* composition, perhaps suggesting that the interaction among impacts was the key regulating factor for this group. In contrast, substrate supply was clearly the strongest driver of *nosZ* composition in these streams. These results suggest that even if these streams were only receiving organic pollutants (i.e., nitrogen, phosphorus, and carbon) rather than both organic and inorganic pollutants from the watershed, there would still be substantial changes to *nosZ* composition.

Based on pairwise correlations, the diversity (both richness and evenness) of *nirK* communities was inversely related to our measure of thermal stress, suggesting that streams with high thermal stress had highly uneven, taxa poor communities. The evenness of *nirK* communities was also inversely related to hydrologic disturbance, suggesting decreasing evenness with increasing disturbance. These findings provide some support for the theory that severe and frequent disturbance may prevent microbial assemblages from progressing beyond early successional stages typically characterized by low diversity (Grime 1974; Pickett and White 1985; Wilkinson 1999). In contrast, *nosZ* diversity did not appear to be correlated with any of our measures of urban impacts.

Based on these results, we conclude that different aspects of the urban stream syndrome control denitrifier community composition versus diversity. Although both aspects of community structure responded to temperature, only composition was related to substrate supply and contamination and only diversity was correlated with hydrologic disturbance. Given that both community composition and diversity can affect ecosystem process rates (Fridley 2001; Hillebrand, Bennett, and Cadotte 2008), studies examining microbial controls on ecosystem functioning need to consider both aspects of community structure. Furthermore, if we had only examined diversity, we might have erroneously concluded that hydrologic disturbance did not have any obvious impact on denitrifier communities.

4.4.2 Nitrite versus nitrous oxide reducers

Study results also demonstrate that both the composition and diversity of nitrite reducing (i.e., *nirK*) versus N_2O reducing (i.e., *nosZ*) denitrifier communities responded to different drivers in these study streams. These results corroborate earlier findings suggesting that the abundances of *nirK* versus *nosZ* denitrifiers in soils responded to different drivers (Bru et al. 2011). Because N_2O is a potent greenhouse gas, the question

of what regulates the ratio of N_2O to N_2 produced by denitrification has been an active field of research (Firestone, Firestone, and Tiedje 1980). These factors are thought to include not only abiotic factors like pH, but also biotic factors like the proportion of the overall denitrifier community having the *nosZ* gene (Philippot et al. 2011). While we did not directly investigate N_2O emissions in this study, our findings provide initial insight on controls of *nirK* versus *nosZ* composition and diversity in streams, which is important, given recent findings suggesting that streams, particularly urban streams, may be sources of N_2O emissions (Beaulieu et al. 2011).

Contamination appeared to have a strong impact on *nosZ* composition; three heavy metals were significantly associated with the ordination of *nosZ* composition. In contrast, *nirK* composition appeared to be unaffected by heavy metals, suggesting that *nosZ* communities may be the more sensitive of the two groups. The same could also be said in terms of their relative responses to hydrology. *nosZ* composition was significantly correlated with storm surge, one of our measures of hydrologic disturbance, while no such relationship was found for *nirK* composition. Both groups responded to at least one measure of temperature. Given that they may be more sensitive and more rare (Bru et al. 2011) than *nirK* denitrifiers, the structure of *nosZ* denitrifier communities may be more likely to limit denitrification, particularly complete denitrification, than that of *nirK* denitrifiers.

4.4.3 What are the direct effects of urbanization on denitrification potential?

Based on SEM results, the direct effects (i.e., pathways not mediated through denitrifier community structure) of urbanization on denitrification potential were positive and driven by increased daily fluctuations in streamwater temperatures (temperature range) and nitrogen (TN) supply. Large riparian buffers appeared to

dampen temperature fluctuations and streams with large temperature fluctuations also tended to have high maximum temperatures. It is unclear why temperature fluctuations per se might stimulate denitrification, but these results are generally consistent with prior findings of increasing denitrification potential with increasing streamwater temperatures (Inwood, Tank, and Bernot 2007; Mulholland et al. 2008).

Increased temperatures may stimulate denitrification potential by increasing the abundance of denitrification enzymes through: 1) increased production per individual denitrifier and/or 2) increased growth rates and, thereby, higher denitrifier abundances. We did not collect information on denitrifier abundances, so we can only speculate as to the actual mechanisms. Future studies could address this uncertainty by using quantitative PCR (Osborn and Smith 2005) to estimate denitrifier abundances in streams along a gradient of urbanization.

Nitrogen supply increased with increasing urbanization intensity, particularly traffic volume, which provides an indication that car exhaust emissions from roads situated close to streams can be a major source of bioavailable nitrogen in urban streams (Bernhardt et al. 2008). Not surprisingly, increased nitrogen availability led to increased denitrification potential, likely a consequence of increased production of denitrification enzymes by denitrifiers. Like temperatures, nitrogen supply may also play an important role in regulating denitrifier growth rates.

Altogether, these results indicate that the direct effects of urban impacts on denitrification potential were positive and large (standardized path coefficients added up to 0.959). Temperature and nitrogen, however, were not the only important drivers of denitrification in these streams. Denitrifier community structure also played an important and contradictory role in regulating denitrification. The effects of denitrifier community structure on denitrification potential were *negative* and approximately

equivalent in magnitude to the direct effect of nitrogen (standardized values were 0.572 for denitrifier community structure versus 0.574 for nitrogen).

The role of temperature and substrate supply in regulating denitrification potential in streams is already well established (Groffman, Dorsey, and Mayer 2005; Inwood, Tank, and Bernot 2007; Mulholland et al. 2008). This study provides a novel perspective by highlighting the critical role that denitrifier community structure can also play in controlling stream denitrification, particularly in the context of increasing watershed urbanization. We do not argue that denitrifier community structure is the only, or even the most important, control of denitrification. Instead, we argue that making the effort to characterize and study the microbial communities actually mediating the process can greatly improve our mechanistic understanding of the functional capacity of ecosystems.

4.4.4 What are the biotic controls on denitrification potential?

Based on SEM results, denitrifier community composition and diversity significantly affect denitrification potential. It is important to note here that results here show that both *nirK* and *nosZ* denitrifiers contributed to explaining estimates of denitrification potential, since both groups produce the enzymes needed to reduce nitrate to N_2O and estimates were based on the amount of N_2O produced over time.

Nitrogen supply shifted the composition of *nirK* communities in a way that stimulated denitrification potential, possibly by selecting for nitrite reducing denitrifiers that are fast-growing and highly productive under high resource conditions – taxa that might be described as r-selected, weedy ruderals by animal and plant ecologists (Grime 1974)). Indeed, there is growing evidence that the r versus K-selected categories commonly used to characterize plants and animals can also be applied to different

bacterial phyla (Fierer, Bradford, and Jackson 2007). Nitrogen availability was also positively associated with *nosZ* evenness, which had a positive effect on denitrification.

Contrary to the negative relationships commonly found between nutrient supply and plant community evenness (Hillebrand, Bennett, and Cadotte 2008), increased nitrogen availability promoted *nosZ* evenness in our study streams. High resource conditions may have reduced competition and, thereby, encouraged a more equitable distribution of abundance. The relationship between evenness and process rates is not well established, with some studies finding a negative and others a positive relationship (reviewed by Hillebrand, Bennett, and Cadotte 2008). A negative relationship might be expected under stable conditions, wherein dominance by the most productive species leads to the highest process rates. On the other hand, a positive relationship might be expected under fluctuating conditions, wherein increased evenness promotes the capacity of the community to adapt quickly to new conditions (Norberg et al. 2001). Consistent with this theory, we found a positive relationship between evenness and function in streams characterized by constant chemical and hydrologic change.

In contrast to nitrogen supply, hydrologic disturbance (storm surge) had a negative effect on *nosZ* evenness. Hydrologic disturbance increased with increasing urbanization intensity (% development), likely as a result of increases in impervious surfaces that can collect and route large amounts of water into stream channels very quickly (Walsh et al. 2005). As discussed earlier, disturbance by scouring flows may have decreased evenness by suspending communities at early successional stages dominated by a few highly tolerant species (Grime 1977; Pickett and White 1985; Wilkinson 1999). Given that nitrogen supply and hydrologic disturbance pushed evenness in different directions and that the absolute value of their standardized pathway coefficients were nearly identical (0.233 versus 0.235, respectively), the net

effect of increased urbanization intensity on *nosZ* evenness was likely small. Yet, if we had not considered *nosZ* evenness, we might have erroneously concluded that hydrology had no clear effect on either denitrifier community structure or function.

The same could also be said for contamination. *nosZ* composition was influenced by lead contamination and nitrogen supply. Lead did not directly affect denitrification or any other measure of denitrifier community structure. Increasing lead concentrations were driven primarily by increasing % development (0.87 standardized pathway coefficient) and secondarily by decreasing traffic volume (-0.27 standardized pathway coefficient). Due to the phasing out of leaded gasoline in the 1990s, the contemporary contribution of lead from vehicle emissions is now negligible (Sutherland 2000), which may explain why our measure of traffic volume, based on 2009 data, was not positively associated with lead. Current major sources of lead in urban streams include sewer and wastewater effluents, storm drain outlets, and soil erosion from residential and industrial areas (Sutherland 2000).

Contrary to *nirK* composition, urban impacts (i.e., increased nitrogen and heavy metal concentrations) appeared to alter *nosZ* composition in a way that limited denitrification potential. High nutrient conditions may lead to the preferential selection of nitrite reducing over N_2O reducing denitrifiers. N_2O reduction is the least energetically favorable step in denitrification (Zumft 1997) and bacteria that 'waste' energy and time carrying denitrification to completion may be at a disadvantage relative to bacteria that stop earlier in the process. If true, such changes to denitrifier community composition may lead to increases in the ratio of N_2O to N_2 produced by denitrification.

Contamination by heavy metals, on the other hand, might select for highly tolerant denitrifiers that are, as a tradeoff, less efficient denitrifiers. That is, tolerance mechanisms are typically energetically costly and accompanied by tradeoff traits that

would otherwise be unfavorable to organism fitness (Grime 1977). Consistent with this theory, multiple metal resistant bacteria in freshwater lakes express fewer enzymes than single metal resistant bacteria (De Souza et al. 2007). This could lead to decreasing availability of N_2O reductase with increasing contamination from urban inputs. Clearly, the impacts of watershed urbanization on denitrifier community structure in streams may have some important consequences for denitrification, both in terms of the amount of reactive nitrogen removed and the amount of N_2O produced.

4.4.5 Conclusions

This study demonstrates that 1) both the composition and diversity of denitrifier communities in streams are affected by watershed urbanization, 2) *nirK* versus *nosZ* denitrifiers respond to urban impacts differently, and 3) both the composition and diversity of denitrifier communities have the potential to be important controls of denitrification potential. Moreover, by highlighting the negative effects that urbanization-driven changes to denitrifier community structure can have on denitrification potential, this study provides a mechanistic explanation for prior findings of decreasing stream denitrification efficiency with increasing watershed urbanization (Inwood, Tank, and Bernot 2007; Mulholland et al. 2008).

While still provisional and of uncertain generality in many respects, our structural equation model was able to capture a large proportion of the variation in denitrification potential among streams. Perhaps more importantly, the model also provided some very interesting, both in the sense of general ecological theory and implications for ecosystem management, initial insights into how the "urban stream syndrome" (Walsh et al. 2005) alters the structure and function of ecologically significant microbial communities like the denitrifiers. We hope these findings will motivate scientists studying urban and non-urban ecosystems alike to consider the microbial

communities that may be mediating how human activities are affecting ecosystem functioning.

4.5 Supplementary Materials

Table 20: Pearson's product-moment correlation coefficients between all temperature variables and denitrification potential (DEA)

	min temp	max temp	mean temp	temp range	Log-DEA
min temp	1.00				0.120
max temp	0.805*	1.00			0.364*
mean temp	0.951*	0.940*	1.00		0.184
temp range	0.120	0.685*	0.404*	1.00	0.462*
Log-max change	-0.030	0.099	-0.020	0.201	0.148

Note: * $p \leq 0.05$. Some temperature variables were $\log_{10}$-transformed prior to analysis.

Table 21: Pearson's product-moment correlation coefficients between all substrate variables and denitrification potential (DEA)

	NO_3	NH_4	TN	TOC	Log-DEA
NO_3	1.00				0.195
NH_4	0.116	1.00			0.282
TN	0.421*	0.202	1.00		0.442*
TOC	-0.357*	0.151	0.205	1.00	0.040
AFDM	-0.181	0.005	0.054	-0.084	0.425*

Note: * $p \leq 0.05$. All substrate values were $\log_{10}$-transformed prior to analysis

Table 22: Pearson's product-moment correlation coefficients between all contamination variables and denitrification potential (DEA)

	Ag	Al	As	Cd	Cr	Cu	Ni	Pb	Log-DEA
Ag	1.00								-0.233
Al	0.175	1.00							-0.266
As	-0.059	0.226	1.00						-0.051
Cd	0.170	0.596*	0.223	1.00					0.085
Cr	0.063	0.662*	0.266	0.309*	1.00				-0.259
Cu	0.206	0.378*	-0.012	0.307*	0.329*	1.00			-0.072
Ni	0.147	0.781*	0.209	0.686*	0.691*	0.222	1.00		-0.176
Pb	0.079	0.724*	0.250	0.827*	0.362*	0.330*	0.636*	1.00	-0.007
Zn	0.170	0.633*	0.211	0.788*	0.392*	0.737*	0.597*	0.729*	-0.010

Note: * $p \leq 0.05$. All heavy metal values were $\log_{10}$-transformed prior to analysis

Table 23: Pearson's product-moment correlation coefficients between all hydrology variables and denitrification potential (DEA)

	incision	transitions	Log-DEA
incision	1.00		-0.042
transitions	-0.010	1.00	0.131
Log-storm surge	0.477*	0.211	0.157

Notes: * $p \leq 0.05$. Some hydrology variables were $\log_{10}$-transformed prior to analysis.

Table 24: Pearson's product-moment correlation coefficients between all denitrifier community structure variables and denitrification potential (DEA)

	nirK					*nosZ*				Log-DEA
	1	2	3	G	R	1	2	3	G	
nirK 1	1.00									-0.27
nirK 2	-0.07	1.00								-0.04
nirK 3	0.02	0.00	1.00							0.24
nirK G	-0.15	0.90*	0.05	1.00						0.13
nirK R	0.08	-0.84*	0.01	-0.92*	1.00					-0.08
nosZ 1	0.03	0.08	-0.08	0.01	0.02	1.00				-0.27
nosZ 2	0.06	0.06	0.15	0.02	-0.01	0.06	1.00			0.04
nosZ 3	0.16	0.01	-0.08	-0.11	0.02	0.13	0.68*	1.00		-0.18
nosZ G	-0.03	0.02	-0.03	-0.11	0.09	0.60*	0.67*	0.40*	1.00	-0.13
nosZ R	0.15	-0.04	-0.03	0.08	-0.09	-0.51*	-0.17	-0.22	-0.90*	-0.02

Note: * $p \leq 0.05$. Numbers after *nirK* and *nosZ* refers to the ordination axis for that respective denitrifier group. G refers to community evenness, while R refers to community richness.

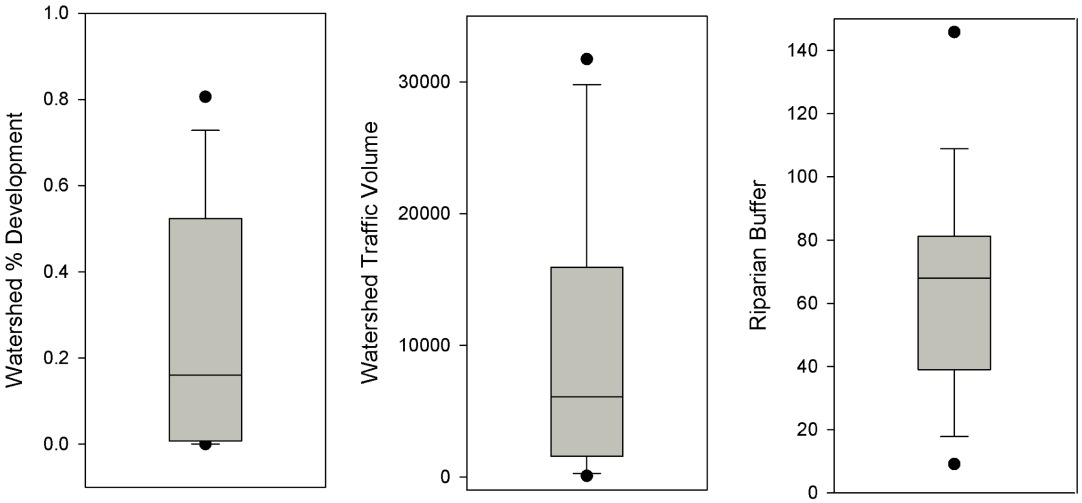

Figure 10: Box plots illustrating quantiles of watershed land use/land cover variables from Table 1. Dots represent the 5th and 95th percentiles.

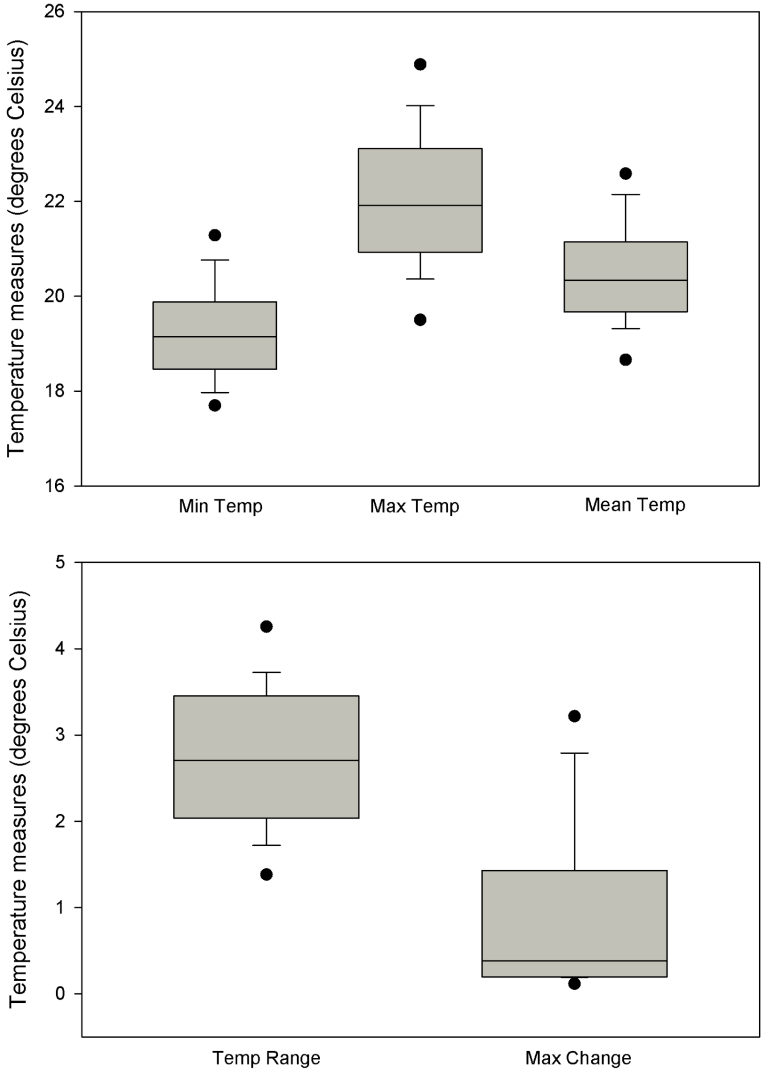

Figure 11: Box plots illustrating quantiles of streamwater temperatures from Table 1. Dots represent the 5th and 95th percentiles.

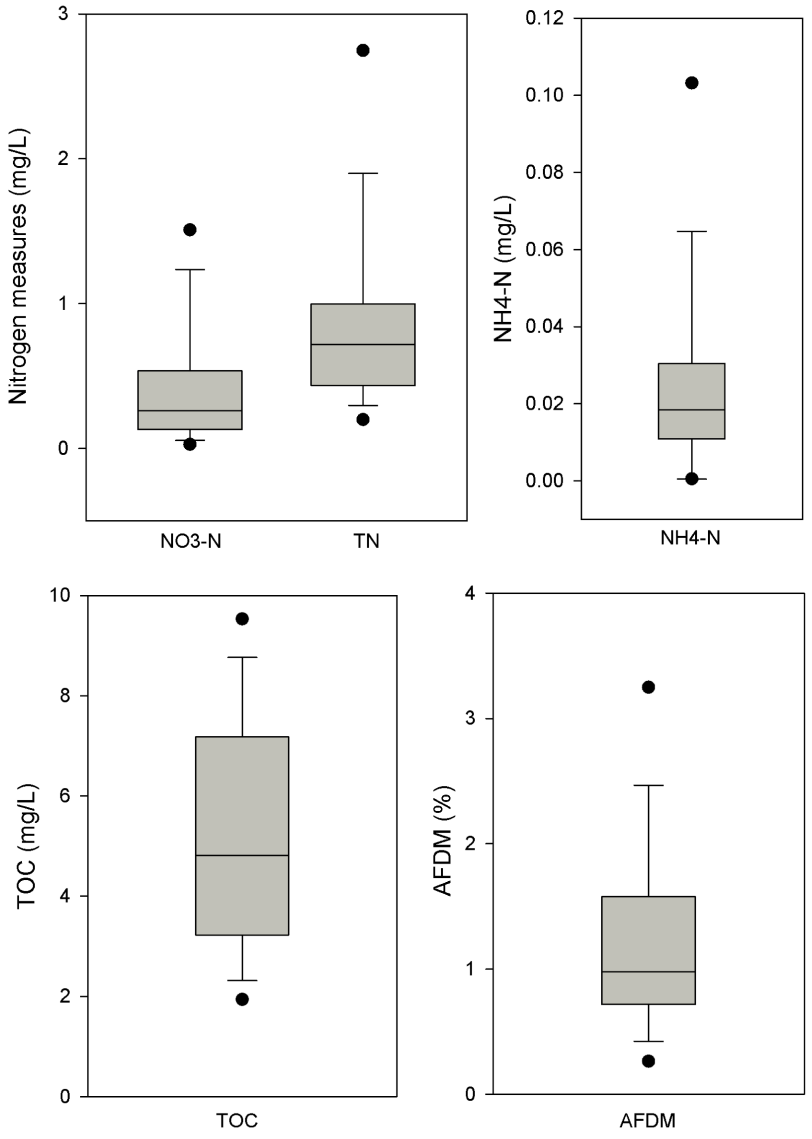

Figure 12: Box plots illustrating quantiles of substrate supply variables from Table 1. Dots represent the 5th and 95th percentiles.

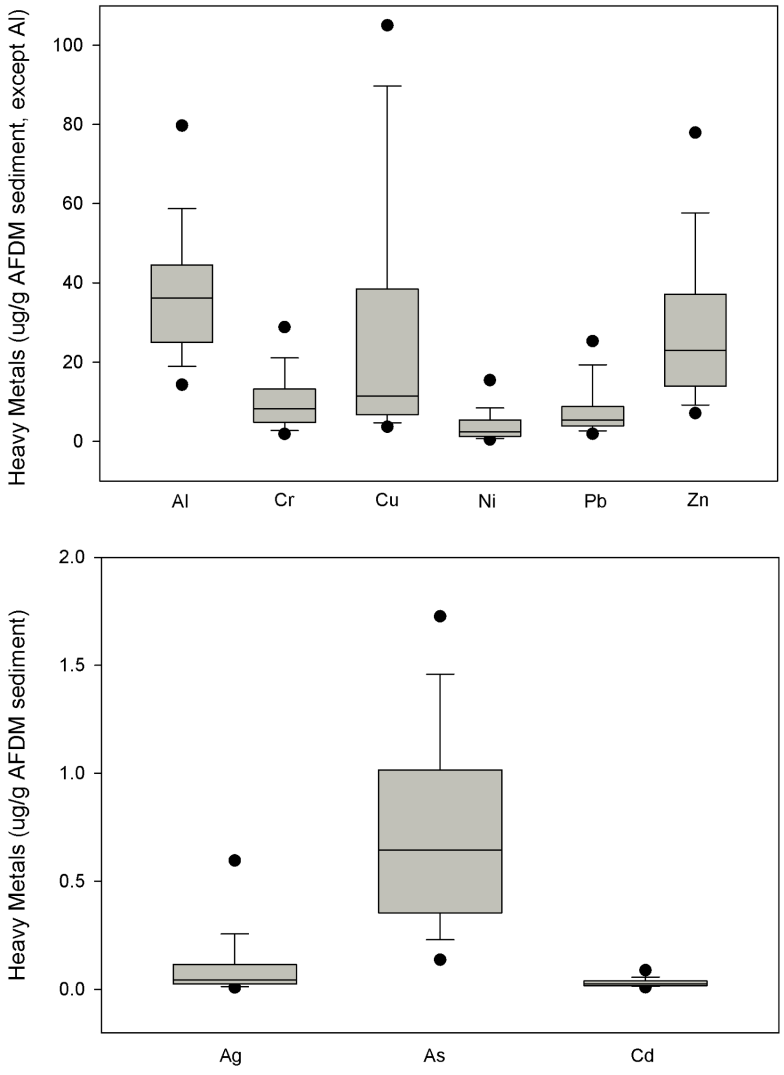

Figure 13: Box plots illustrating quantiles of contamination variables from Table 1. Dots represent the 5th and 95th percentiles. Aluminum concentrations are presented as mg/g AFDM sediment.

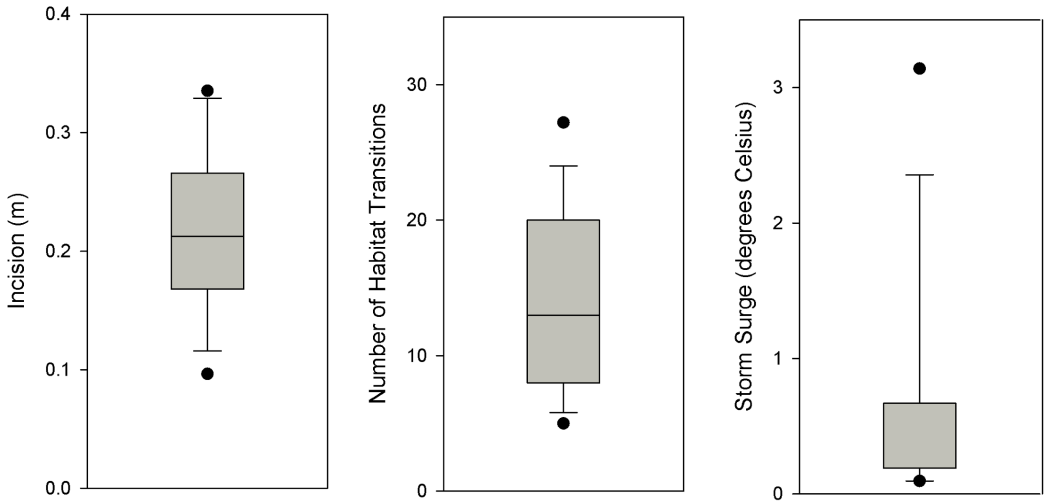

Figure 14: Box plots illustrating quantiles of hydrology variables from Table 1. Dots represent the 5th and 95th percentiles.

5. Conclusions

This dissertation highlights the key role microbial communities play in mediating how anthropogenic disturbances affect ecosystems and their ability to provide ecosystem services. While it is difficult and perhaps futile to even contemplate conserving or protecting microbial species like we have done for more charismatic taxa like the panda, we argue that making the effort to at least characterize the microbial communities that actually perform the ecosystem services we care about can greatly improve our mechanistic understanding of and, therefore, ability to predict how ecosystem functioning might respond to further environmental degradation. We cannot expect to be able to effectively manage and restore ecosystems without such knowledge.

In the specific case of the denitrifiers and denitrification in streams, this dissertation demonstrates that watershed urbanization and its associated impacts on streamwater temperatures, substrate supply, contamination, and hydrology all have an effect on some aspect of denitrifier community structure in streams. Urbanization-driven changes to denitrifier community structure can, in turn, constrain the capacity of stream ecosystems to remove harmful excess reactive nitrogen and possibly also their ability to carry denitrification to completion. Furthermore, loss of denitrifier diversity may also limit the capacity of denitrifier communities to resist changes to function in response to disturbance, suggesting that the functioning of urban streams may be more vulnerable to further environmental degradation that of more pristine streams.

In summary, this dissertation provides a concrete example of when and how microbial community structure 'matters' from a functional standpoint and should thus be explicitly considered in models of microbe-mediated ecosystem processes. Given the molecular tools we now have for describing complex microbial communities, we can no longer blindly assume that anthropogenic disturbances do not substantially alter the

structure and function of the "unseen majority" that drive Earth's biogeochemical cycles (Falkowski, Fenchel, and Delong 2008; Whitman, Coleman, and Wiebe 1998). We hope that this dissertation demonstrates how theories and concepts from decades of ecological research on 'macrobes' can help the relatively young field of microbial ecology develop to a more mature, predictive science that will help improve our ability to address the pressing environmental problems of our time.

5.1 Future directions for my personal research

I am broadly interested in continuing to conduct research on the causes and functional consequences of microbial community structure, particularly under realistic scenarios of environmental degradation, whether they be a result of land use change, climate change, or any other anthropogenic driver. My dissertation focused specifically on denitrifiers and denitrification in streams. I would be interested in expanding my research to other terrestrial or aquatic ecosystems and to other functional groups of microbes, particularly those that are ecologically important, such as nitrifying bacteria, nitrogen fixing bacteria, methanogens, and methane oxidizers. That is, my main interest is in the fundamental links between structure and function for microbial communities and I am open to using different model systems to study these links. I also believe that both observational and experimental approaches are necessary to develop a better understanding of these topics. Ultimately, my hope is that my work will provide information that is useful to ecosystem management and restoration efforts.

5.2 Future directions for microbial ecology

The observational, experimental, and statistical approaches used in this dissertation provide a general blueprint for studying how functional groups other than the denitrifiers may be responding to anthropogenic disturbances. For instance, a better understanding of how methanogens versus methane oxidizers might be responding to

climate change, particularly increased temperatures and altered precipitation, is urgently needed to improve our ability to predict how methane concentrations might change in the coming years. As molecular technologies continue to advance at a rapid pace, the temptation to favor increasingly complex (and expensive) molecular methods over simpler methods of describing microbial communities needs to be tempered by a theoretical foundation that places as much premium on sound hypotheses and elegant experimental designs as on using the newest technologies. Microbial ecologists still have a great deal to learn from biogeochemists, ecosystem ecologists, landscape ecologists, and community ecologists and I firmly believe that the successful future of microbial ecology relies heavily on increased dialogue amongst the different ecological disciplines.

Appendix A

Table 25: Substrate supply and overall bacteria TRFLP data

Stream	Sampling Date	DEA (ng N g^{-1} hr^{-1})	Nitrate (mg L^{-1})	Total Organic Carbon (mg L^{-1})	Overall Bacteria # of OTUs	Overall Bacteria NMS Axis 1	Overall Bacteria NMS Axis 2	Overall Bacteria NMS Axis 3
Mud Creek	1	52.61	0.052	5.336	-	-	-	-
Mud Creek	2	49.52	0.159	5.511	-	-	-	-
Mud Creek	3	217.60	0.121	3.477	40	- 0.268	0.008	0.331
Stony	1	164.24	0.163	5.593	-	-	-	-
Stony	2	140.81	0.250	4.118	-	-	-	-
Stony	3	297.05	0.270	4.064	47	- 0.093	0.271	0.222
Stony	4	222.01	0.117	2.915	70	- 0.314	0.039	-0.042
Lower Mud	1	93.06	0.190	6.255	-	-	-	-
Lower Mud	2	72.57	0.060	4.999	-	-	-	-
Lower Mud	3	62.25	0.184	4.605	44	- 0.159	0.348	- 0.042
Pott's	1	40.54	0.124	4.987	-	-	-	-
Pott's	2	96.78	0.017	3.493	-	-	-	-
Pott's	3	210.64	0.102	4.925	48	0.044	0.257	- 0.287
Pott's	4	165.49	0.089	5.297	68	- 0.290	- 0.129	- 0.035
Upper Mud	1	211.57	0.142	8.415	-	-	-	-
Upper Mud	2	134.45	0.107	5.376	-	-	-	-
Upper Mud	3	292.67	0.059	5.143	58	0.221	- 0.154	0.117
Upper Mud	4	126.96	0.200	6.226	53	- 0.024	- 0.401	0.102
Cemetery	1	115.67	1.446	1.775	-	-	-	-
Cemetery	2	555.61	1.401	1.298	-	-	-	-
Cemetery	3	561.62	1.706	1.638	33	0.387	0.403	- 0.036
Cemetery	4	531.89	1.191	3.889	53	- 0.178	0.027	- 0.321
Ellerbee	1	107.50	0.287	8.300	-	-	-	-
Ellerbee	2	104.13	0.127	7.510	-	-	-	-
Ellerbee	3	110.96	0.232	7.061	45	0.110	- 0.015	0.404
Ellerbee	4	67.58	0.214	7.618	64	- 0.159	- 0.275	- 0.202
Goose	1	232.60	0.018	35.525	-	-	-	-
Goose	2	95.42	0.354	10.419	-	-	-	-
Goose	3	552.00	0.225	7.647	40	0.467	- 0.124	0.014
Goose	4	167.24	0.201	7.084	50	0.256	- 0.252	- 0.226

Table 26: Denitrifier TRFLP data

Stream	Sampling Date	*nirK* Denitrifiers				*nosZ* Denitrifiers			
		# of OTUs	NMS Axis 1	NMS Axis 2	NMS Axis 3	# of OTUs	NMS Axis 1	NMS Axis 2	NMS Axis 3
Mud Creek	1	79	- 0.44	0.02	- 0.01	88	0.40	0.15	- 0.28
Mud Creek	2	53	- 0.41	- 0.25	0.12	82	- 0.11	0.38	- 0.21
Mud Creek	3	101	0.03	0.30	0.25	87	- 0.07	- 0.14	- 0.45
Stony	1	74	- 0.39	0.13	0.16	84	0.31	- 0.08	- 0.35
Stony	2	104	- 0.24	0.29	0.24	122	- 0.07	0.20	- 0.35
Stony	3	101	- 0.11	0.17	0.34	127	- 0.33	- 0.19	- 0.17
Stony	4	105	- 0.04	0.39	0.08	132	- 0.16	- 0.33	- 0.23
Lower Mud	1	61	- 0.37	0.14	- 0.27	92	0.41	- 0.15	- 0.15
Lower Mud	2	87	- 0.22	0.32	- 0.13	77	0.09	0.38	- 0.17
Lower Mud	3	79	0.26	0.03	0.34	99	- 0.29	- 0.03	- 0.32
Pott's	1	55	- 0.33	- 0.10	- 0.33	106	0.41	- 0.03	0.02
Pott's	2	58	- 0.30	- 0.30	- 0.15	84	- 0.26	0.29	- 0.13
Pott's	3	45	- 0.08	- 0.40	- 0.28	116	- 0.38	- 0.05	- 0.03
Pott's	4	109	0.04	0.05	0.25	108	- 0.29	- 0.29	0.03
Upper Mud	1	72	0.01	- 0.01	- 0.43	91	0.23	0.11	0.42
Upper Mud	2	92	- 0.01	0.17	- 0.33	96	0.04	0.38	0.08
Upper Mud	3	86	0.11	0.28	- 0.20	60	0.24	- 0.49	- 0.05
Upper Mud	4	84	0.23	0.29	- 0.18	125	- 0.07	- 0.34	0.04
Cemetery	1	72	- 0.22	- 0.20	0.35	91	0.46	0.08	- 0.02
Cemetery	2	68	0.01	- 0.33	0.36	93	- 0.30	0.26	0.19
Cemetery	3	82	- 0.02	- 0.38	0.11	131	- 0.34	- 0.06	0.14
Cemetery	4	71	0.15	- 0.40	0.24	132	- 0.22	0.02	0.30
Ellerbee	1	84	0.32	0.29	0.14	85	0.38	- 0.14	0.30
Ellerbee	2	74	0.44	0.12	0.06	100	- 0.19	0.34	0.03
Ellerbee	3	89	0.03	0.15	- 0.02	111	0.03	- 0.36	0.26
Ellerbee	4	48	0.44	- 0.20	0.15	120	0.00	- 0.23	- 0.10
Goose	1	81	0.25	- 0.22	- 0.34	98	0.37	0.26	0.19
Goose	2	86	0.21	- 0.31	- 0.13	93	- 0.08	0.35	0.26
Goose	3	65	0.35	0.06	- 0.27	136	- 0.19	- 0.20	0.33
Goose	4	96	0.31	- 0.11	- 0.09	130	- 0.04	- 0.09	0.41

Appendix B

Table 27: TRFLP data for *nirK* denitrifiers

Stream	Replicate	No. of OTUs	Evenness (G)	NMS Axis 1	NMS Axis 2
Forest	1	51	0.729	0.514	- 0.171
Forest	2	40	0.781	0.491	0.063
Urban	1	8	0.964	- 0.456	- 0.036
Urban	2	36	0.850	- 0.157	0.413
Urban	3	6	0.963	- 0.392	- 0.269

Appendix C

Table 28: TRFLP data of *nirK* and *nosZ* denitrifiers

Stream	*nirK*		*nosZ*	
	No. of OTUs	Evenness (G)	No. of OTUs	Evenness (G)
AB	59	0.906	90	0.940
B1	82	0.802	143	0.872
B2	60	0.860	52	0.963
B3	41	0.866	118	0.907
CM	77	0.891	131	0.904
DS07	31	0.903	108	0.934
DS13	27	0.910	96	0.935
DS31	137	0.689	108	0.925
DW05	116	0.758	100	0.920
DW07	94	0.751	67	0.957
DW42	57	0.899	86	0.908
E01	122	0.724	54	0.971
E04	97	0.825	82	0.943
E07	45	0.853	50	0.953
E11	93	0.784	74	0.953
E12	54	0.849	12	0.987
E13	59	0.831	78	0.960
E15	85	0.769	87	0.954
E16	69	0.806	166	0.907
FH	103	0.753	75	0.935
GC	64	0.839	138	0.890
LM	84	0.740	102	0.927
M05	36	0.883	109	0.923
M08	63	0.861	103	0.928
M14	78	0.856	89	0.934
M15	69	0.872	144	0.894
M16	57	0.844	135	0.914
M20	9	0.978	70	0.953
M21a	26	0.904	106	0.940
M24	103	0.791	143	0.898
M25	83	0.820	134	0.898

Table 28 continued

Stream	*nirK*		*nosZ*	
	No. of OTUs	Evenness (G)	No. of OTUs	Evenness (G)
M29	64	0.804	113	0.919
M32	84	0.787	59	0.962
M38	98	0.754	93	0.941
M39	74	0.811	77	0.943
M46	5	0.978	73	0.944
M51	63	0.848	146	0.899
M58	76	0.874	21	0.980
M68	118	0.714	73	0.976
M71	99	0.718	92	0.946
M75	88	0.796	128	0.917
MT	94	0.759	85	0.931
NG	70	0.853	119	0.919
PN	41	0.884	114	0.921
PHY	83	0.774	2	0.934
RB	12	0.954	121	0.927
SA	60	0.900	101	0.935
ST	90	0.755	125	0.889
UM	81	0.771	55	0.967

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Biography

Si-Yi Wang was born in 1982 in Taipei, Taiwan. She moved to Hong Kong at a young age and attended boarding school in Groton, Massachusetts (USA) between 1995 and 2000. She spent a year working in Malaysia and studying in Beijing, China before enrolling at Wellesley College in Wellesley, Massachusetts (USA) in 2001. She graduated from Wellesley College in 2004 with a Bachelor of Arts in Environmental Studies.

Si-Yi, who is also known as Jenny to friends and family, enrolled at Duke University in 2006 after spending two years working in Cambridge, Massachusetts (USA) for an environmental consulting firm as a research analyst. During her time at Duke University, Si-Yi received grants from Wellesley College, Society of Wetland Scientists, Sigma Xi, and the National Science Foundation to support her research. She has also presented research at several scientific meetings and received an award from the American Society of Limnology and Oceanography for an oral presentation at the 2011 meeting held jointly with the North American Benthological Society.

Si-Yi is a member of the American Society of Limnology and Oceanography, British Ecological Society, Ecological Society of America, International Society of Microbial Ecology, and North American Benthological Society.