UTILIZATION OF DIFFERENT FORMS OF NITROGEN BY HETEROTROPHIC BACTERIA UNDER VARYING ORGANIC CARBON CONCENTRATIONS: FROM ISOLATES TO COMMUNITIES

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By
Suchismita Ghosh
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CHAPTER 1: GENERAL INTRODUCTION

Microbial Response To Nutrients

Microbial transformations are important for carbon, nutrient, and energy flow within an ecosystem (Roling et al. 2007) and for the sustenance of all forms of life on this planet (Fenchel 1998, Peay et al. 2008). Microbes serve as a basic trophic layer (Wardle 2002) because they transform organic and inorganic molecules into biologically utilizable forms (Madigan et al. 2006). As a result of this profound impact on food webs and nutrient cycles, metabolic activity of microbes (like respiration) is often considered to represent the functional activity of the ecosystem (Ducklow 2007).

However, microbial communities are comprised of many taxa (Torsvik et al. 2002, Fenchel and Findlay 2004), and most of these organisms are extremely difficult to culture (Amann et al. 1995). In order to maximize the cultivable fraction of natural microbial communities, improved methods of cultivation and culture media have been devised to mimic natural environment in terms of nutrients (Zengler et al. 2005). Yet, most microbes are not cultivable by standard culturing techniques (Hugenholtz 2002). Although, these organisms remain viable in their natural environment, they fail to grow under laboratory conditions and referred to as “viable but non-culturable” (VBNC) (Oliver 2005). Cultured microorganisms constitute <1% of all microbial species (Hugenholtz 2002).

Molecular based methods on 16S rRNA genes reveal the presence of highly diverse and widely distributed candidate bacterial divisions (such as BRC1, OP10, OP11,
SC3, TM7, and WS2) that have no cultured representatives and are only known by their molecular sequences (Schloss and Handelsman 2004). Advancements in molecular-based techniques have enabled researchers to get a glimpse of complex patterns that exist at the microbial scale (Zak et al. 2006, Hirsch et al. 2010). Although, metabolic activity of microbes occurs at a micrometer scale (Young et al. 2008), in aquatic ecosystems it can influence nutrient pools and organisms in the range of micrometers to kilometers (Ducklow 2007).

Among microbes, bacteria are considered to be one of the diverse and dominant forms (Curtis et al. 2006; Whitman et al. 1998) and play a vital role in driving essential biogeochemical cycles (Allan 1995). An important trait of bacterial communities in any ecosystem is their ability to exploit different types of resources and these traits can affect biodiversity and ecosystem function (Naeem et al. 1999). However, the role of bacterial diversity in driving such ecosystem functions has been neglected (Hillebrand and Matthiessen 2009) and ecologists are beginning to realize the importance of biotic and abiotic factors in shaping microbial diversity and their role in biogeochemical models (Allison and Martiny 2008).

**Nitrogen and Its Utilization By Bacteria**

Nitrogen (N) is an essential biological molecule and affects ecosystem productivity (Luo et al. 2004). N can be limiting in some streams (Grimm and Fisher 1986), but excess N can have detrimental effects, like eutrophication. Understanding the nitrogen cycle is particularly important in streams because anthropogenic activity, such as
agriculture, has increased inputs of biologically reactive nitrogen to the environment (Kemp and Dodds 2001; Royer et al. 2004). Harmful algal blooms and formation of dead zones in lakes and oceans are consequences of the downstream transport of these nutrients.

The bioavailability of N in an ecosystem depends on conversion of nitrogen containing compounds into utilizable forms. Bacteria can convert a wide range of N compounds into forms that vary in their availability to other organisms (Madigan et al. 2006). While parts of the N cycle involve abiotic processes (e.g., lightening causing oxidation of N₂ to NO₃), biotic conversion via bacteria largely drives essential transformations.

The total N pool in streams is comprised of various forms of dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN). The most abundant forms of DIN in most streams are nitrate and ammonium (Peterson et al. 2001). DON constitutes a substantial proportion of stream N (Perakis and Hedin 2002) and can originate from both external sources, like terrestrial inputs (allochthonous), and internal sources (autochthonous), like in-situ release by algae. DON is composed of a plethora of compounds ranging from low molecular weight (LMW) simple compounds, like urea and amino acids, to high molecular weight (HMW) compounds composed of complex tannins, alkaloids, and humic compounds. Bacteria can utilize both the LMW labile fraction (Keil and Kirchman 1993; Middelboe et al. 1995; Middelburg and Nieuwenhuize 2000) and the HMW refractory fraction (Stepanauskas et al., 2002; Seitzinger et al. 2002). Although DON can contribute to eutrophication (Seitzinger and Sanders 1997;
Stepanauskas et al. 2002) and serve as a N source, research on DON and its fate in freshwater ecosystems, including streams, is relatively limited (Kaushal and Lewis 2005; van Kessel et al. 2009).

**Links Between C and N Metabolism**

DON includes both carbon (C) and N, thus C and N metabolism can be coupled. Examining the mechanism of microbial cycling of DON and DOC is important to our understanding of energy and material transfer between trophic levels. DON serves not only as a source of N for the microbial community but also as a source of C. DOC and DON can be either cycled at similar rates (Qualls and Haines 1992) or at different rates (Wiegner and Seitzinger 2001). In addition, N dynamics in streams can be affected by availability of C (Bernhardt and Likens 2002) and rates of N transformation can be influenced by quality and quantity of organic matter (Seitzinger 1994; Jones et al. 1995). Specific N transformations rely on oxidizable C supplies and thus depend on the availability of DOM (Paerl 1993; Seitzinger 1994).

Most research has focused on the N dynamics (Grimm et al. 1981; Triska et al. 1984; Mulholland et al. 2000) or C dynamics (Fisher and Likens 1973; McDowell and Fisher 1976; Meyer and Tate 1983), and only a few studies have addressed both simultaneously (Tank and Webster 1998; Bernhardt and Likens 2002). As DON serves as a source of both C and N, it is likely to be a preferred C and N source for heterotrophic bacteria under low organic C. Since C and N cycles are tightly linked in streams
(Bernhardt and Likens 2002), it is important to understand the influence of alterations in DOM quantity and quality on N dynamics in aquatic ecosystems.

Research Goals and Dissertation Outline

The central goal of my dissertation research is to examine the role of heterotrophic bacteria in driving organic and inorganic N dynamics under varying carbon concentrations. In this study, I tracked patterns of N utilization by heterotrophic bacteria to improve our knowledge about factors that influence bacterial community structure and functional response. Bacterial utilization of N was studied at increasing levels of complexity; as individual isolates, in controlled laboratory microcosms and in natural communities of stream sediment. My research is presented within three chapters; the goals and hypotheses of each chapter are described below.

Chapter 2: Patterns of N utilization by heterotrophic bacteria from streams

The goal of this study was to identify heterotrophic bacteria, isolated from different sole-source N enrichments that differ in their pattern of utilization of various forms of N. In streams, different bacterial taxa may utilize different components of the total N pool. In general, heterotrophic bacteria utilize a wide range DON and DIN sources for their growth, including dissolved free amino acids (Crawford et al. 1974), nucleic acids (Paul et al. 1989), proteins and combined amino acids (Hollibaugh and Azam 1983; Coffin 1989), and ammonium (Wheeler and Kirchman 1986). The ability of heterotrophic bacteria to preferentially utilize one form of N over another or specialize on different sources of N compounds could lead to resource partitioning and perhaps influence the coexistence of bacterial species in natural communities. Also, the fate of
organic and inorganic N in streams can be influenced by bacterial utilization of organic N over inorganic forms.

For this study, sediment samples were collected from three study streams; two in Indiana and one in Ohio. Sediments were enriched in minimal media with seven different N amendments and in a complex medium. Bacteria isolated from these enrichments were grown in 12 different sole-source N compounds. PCR amplification of 16S rDNA of each bacterial isolate followed by sequencing was carried out to identify bacteria that varied in their pattern of N utilization. The hypothesis for this chapter was that bacterial isolates will vary in their growth on various organic vs. inorganic forms of N; the difference in their ability to use various N compounds will categorize them as generalists vs. specialists which will be a function of the taxonomic identity of the organism.

Chapter 3: Impacts of labile organic C concentration on organic and inorganic N utilization by stream bacteria

C availability has a strong influence on N dynamics (Paerl 1993; Seitzinger 1994). Bacterial biomass can be controlled by available C (Bott et al. 1984), which in turn can affect N-demand. Bacterial metabolism of N is dependent on several factors including C availability as well as concentrations of inorganic and organic N (Strauss et al. 2002; Bernhardt and Likens 2002; Kaushal and Lewis 2005). Despite the tight linkage between C and N cycles in streams (Bernhardt and Likens 2002), there has been little research on the role of C availability in the bacterial preference for organic vs. inorganic N. The goal of this study was to understand the impact of C availability on uptake of different N forms (DIN and DON) by stream heterotrophic bacterial communities.
In this study, bacterial communities (that had colonized glass beads incubated in a stream) were treated with varying DOC, DON and DIN concentration in controlled laboratory microcosms. Since DON is diverse with varying bioavailability, three DON sources were used: humic matter, bacterial protein, and algal exudate. Apart from DOC and DON uptake, bacterial response to these treatments was measured as alterations in abundance (based on DAPI counts), community structure and composition (based on T-RFLP of 16S rRNA genes). I hypothesized that bacteria will preferentially utilize organic N under low labile C concentration, however, DIN would be preferred in treatments with high labile C concentration. In addition, I predicted responses to the varying C and N conditions would vary among DON sources based on differences in lability.

Chapter 4: Response of sediment bacterial communities to temporal variation in the dissolved organic matter pool of an agriculturally impacted stream

DOM is often categorized into coarse fractions: high molecular weight or recalcitrant and low molecular weight or labile. Seasonal and hydrologically driven alteration in DOM affects the concentration of these fractions in streams (Fisher and Likens 1973; McDowell and Fisher 1976; Meyer et al. 1998). Bacteria communities in stream ecosystems are impacted by DOM quality, quantity, source and type (Koetsier et al. 1997; Leff, 2000; Leff and Meyer 1991). Properties of the bacterial community, such as respiration, extracellular enzyme activity (Sinsabaugh et al. 1997), biomass, etc., can be affected by dissolved inorganic nutrients and organic matter in streams which
influences community structure and function (Stelzer et al. 2003; Tank and Webster 1998).

Knowledge about the impact of seasonal variation in DOM concentration and source on N processing in streams is limited (Bernhardt and Likens 2002). An understanding of the effects of variability in DOM concentration and quality on bacterial communities can help with predicting changes in ecosystem function as result of alteration in environmental conditions.

The objective of this study was to determine bacterial functional (enzymatic activity) and structural response (community structure based on T-RFLP of 16S rRNA genes) to variation in DOM concentration in an agriculturally impacted stream over the course of a year. Water and sediment samples were collected from the study stream on six dates and routine physicochemical variables were measured. Humic and non-humic fractions of the DOM pool were separated via ion-exchange column chromatography. Aquatic and benthic chlorophyll a (chl a) concentrations were determined and bacterial responses (abundance, community structure) to alterations in the DOM were examined. Functional response to changes in DOM pool was estimated via 5 extracellular enzyme activity; namely, α-glucosidase (AG), β-glucosidase (BG), N-acetyl glucosaminidase (NAG), leucine amino-peptidase (LAP) and lacase (LAC). I predicted that stream heterotrophic bacterial communities would respond to changes in DOM quality and concentration by alteration in community composition that, in turn, will alter extracellular enzyme activity.

Chapter 5: Synopsis
This chapter summarizes and compares all observations and explores implications of my dissertation in a broader context. Anthropogenic activities have altered biogeochemical cycles. In order to understand the mechanism behind altered ecosystem function, stream biogeochemists need to quantify how an ecosystem functions under direct (nutrient cycles) and indirect influences (interactions with other nutrient cycles). The central theme of this dissertation research was to address knowledge gaps related to C and N dynamics in aquatic ecosystems. To investigate the key role played by microbes in N utilization, a molecular-based approach (T-RFLP, sequencing) was adopted along with functional assays and applied under varying environmental conditions.
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CHAPTER 2: PATTERNS OF NITROGEN UTILIZATION BY HETEROTROPHIC BACTERIA FROM STREAMS

Abstract

Identification of bacteria based on molecular methods, like 16S rDNA sequencing, has the potential to yield a wealth of information related to prokaryotic diversity. Knowledge about bacterial patterns of resource utilization is important in understanding essential biogeochemical cycles. The nitrogen cycle in aquatic environment constitutes an array of complex microbially-mediated transformations. In streams, different species of bacteria may utilize different components of the total nitrogen pool. In this study, utilization of organic and inorganic forms of nitrogen by stream heterotrophic bacterial isolates was examined. Sediment samples were collected from three study streams; two agriculturally impacted streams in Indiana and one forested stream in Ohio. Sediments were enriched in minimal media with 7 different nitrogen enrichments and in a complex media. A total of 265 bacterial isolates were obtained from these enrichments and were further subjected to 12 different sole source nitrogen compounds. Results revealed variation in patterns of bacterial N utilization, possibly driven by selection pressure, depending on the microenvironment (enrichments) they were isolated from. PCR amplification of 16S rDNA of each bacterial isolate followed by sequencing, identified bacteria that varied in

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their patterns of N utilization. However bacterial functional response (generalist vs specialist) were unrelated to their taxonomic identity.

**Introduction**

In streams, concentrations and forms of bioavailable nitrogen (N) are determined by inputs from external sources and cycling of nitrogenous compounds within the ecosystem. The resulting total nitrogen pool is comprised of various forms of DON and DIN that vary in bioavailability (McCarthy and Carpenter, 1983; Wheeler and Kokkinakis 1990; Sell and Overbeck 1992; Brookshire et al. 2005). Heterotrophic bacteria utilize a wide range DON and DIN sources, including dissolved free amino acids (Crawford et al. 1974), nucleic acids (Paul et al. 1989), proteins and combined amino acids (Hollibaugh and Azam 1983; Coffin 1989), nitrate (Wheeler and Kokkinakis 1990) and ammonium (Wheeler and Kirchman 1986). The ability of heterotrophic bacteria to preferentially utilize one form of N over another or specialize on different sources of N compounds could lead to resource partitioning and influence the coexistence of bacterial species in natural communities as well as impacting the fate of organic and inorganic N in streams.

Among heterotrophic bacteria there may be a large degree of functional redundancy (Allison and Martiny 2008). However, differences among bacterial taxa in the forms of N utilized may allow resources to be partitioned (Tilman 1982). One manifestation of these taxon-specific functional attributes is the classification of bacteria into ‘generalists’ and ‘specialists’ (Futuyma and Moreno 1988). Some studies suggest
that natural microbial communities are composed of an array of species with varying degrees of ecological specialization (generalist and specialist species) (Futuyma and Moreno 1988). Assemblages of specialists or generalists exhibit different performances across the range of environmental conditions encountered in a fluctuating environment (sensu Kassen, 2002). Although resource utilization differs among taxa, there is uncertainty about the taxonomic scale at which microbes share common responses to environmental variables (Philippot et al. 2010).

In this study, we tested the hypothesis that among heterotrophic bacteria there are differences in the breadth of N compounds utilized and, consequently, some are specialized to use a narrow range of N compounds while others are generalists able to use a broad range of compounds. In addition, we hypothesized that these functional classifications were related to taxonomic affinity with organisms of some taxa more likely to be specialists than others.

To test our hypotheses, sediment samples were collected from three streams (two in Indiana and one in Ohio) and enriched in minimal media with 8 different nitrogen amendments and in nutrient broth (complex medium). Enrichments were incubated for different lengths of time to isolate fast and slow growers. Bacteria isolated from these enrichments were grown in various sole source nitrogen compounds and their growth rates determined using spectrophotometry. 16S rRNA genes were sequenced to identify bacteria that varied in their pattern of N utilization.

Methods
Sediment samples were collected from a forested site along the West Branch of the Mahoning River (WBM), located near Ravenna, OH in Portage County (Olapade and Leff, 2005). WBM is relatively undisturbed with approximately 281 km² of drainage area. Sycamore Creek (SYC) located in Morgan County, 44 km southwest of Indianapolis is a 3rd-order forested stream. This stream flows through Bradford Woods and 57% of the land cover is forested (Baxter et al. 2012). Sediment samples were collected from the forested area of the watershed. The final stream sampled was Sugar Creek near Shirley, IN (SS); a 2nd-order stream in Hancock County, 60 km east of Indianapolis. Primary land use (75%–87%) in Sugar Creek is agriculture (Baker et al. 2006, Lathrop 2006, Stone and Wilson 2006). Use of fertilizers along with herbicides and pesticides are common farming practices in the Sugar Creek watershed. Sugar Creek and Sycamore Creek in Indiana experience seasonal changes in carbon (C) and N concentrations. A recent study by Baxter et al. (2012) revealed increased concentration of nitrate during summer with significantly higher concentration in Sugar Creek (about 4.8 mg/L) as compared to Sycamore Creek (about 1.9 mg/L). However, the concentration of nitrate was low in autumn and was similar in both streams. Unlike nitrate, dissolved organic carbon (DOC) concentrations were high in autumn (approximately 3.5 mg/L) with no significant difference between the streams.

Sediments samples were collected from five sites (5 m apart) along a 25 meter reach of each stream using sterile 50ml centrifuge tubes that were plunged into the sediment to a depth of 3–4 cm. Sediments were collected from the center, the right and left
bank at each site and samples from all the 5 sites were pooled to obtain a representative, composite sample for each stream.

In the laboratory, sediment samples (5 g) were incubated in M9 minimal media with 8 different sole-source N compounds: nitrate, ammonium, urea, glycine, tryptophan, a mixture of different N compounds (ammonium + nitrate + urea + glycine + tryptophan), and bacterial protein (undefined cellular extract) as well as in nutrient broth (complex medium). One set of enrichments was incubated at 25°C for 24 hours (to isolate fast growing bacteria) while another set was incubated for 72 hours (to isolate slow growing bacteria). Samples from each enrichment were used to inoculate plates with the same enrichment medium amended with agar. Two hundred and sixty five colonies with distinctly different morphology (based on shape, size, color and consistency) were selected for further study.

Utilization of 12 different N compounds [nitrate, ammonium, urea, glycine, proline, tryptophan, bacterial protein, peptidoglycan, nucleic acid (purified DNA), algal exudate, putrescine (polyamine), humic matter] by each bacterial isolate was carried out in 96 well microtitre plates. Each sole-source N media had the same concentration of N (94 mM as in standard minimal media) (Maheswaran and Forchhammer 2003). Bacterial cultures were inoculated in broth medium (same composition as the respective enrichment medium) and incubated for 24-48 hours. After the cultures reached a specific optical density (0.4), they were centrifuged and washed (5 times) with N-free minimal media and diluted 1:10 with the N-free minimal media to minimize transfer of N to the 96 well test plates. Washed cultures were transferred to the wells of the plate such that the
inoculant comprised only 10% of the final volume. Plates were incubated at room
temperature for 6 days and optical density (O.D.) was measured at 600nm every 12 hours
for the first 48 hours and every 24 hours for the remaining 4 days. Treatments were
carried out in triplicate for each isolate.

OD values in the range of 1.5-0.5 were considered to represent high bacterial
growth while values < 0.5 represented low or no growth. Bacteria were broadly
categorized into generalists and specialists based on their growth in each of the different
N compounds. For the purpose of this study, high bacterial growth in more than 60% of
the N compounds tested were considered generalists and those that grew in less than 60%
of the compounds were designated as specialists. Bacterial growth rates were calculated
from OD\textsubscript{600} values recorded at the different time points.

Isolates were subjected to PCR amplification of the 16S rRNA gene followed by
sequencing. Genomic DNA was extracted from bacteria using the CTAB method
followed by phenol:chloroform extraction and ethanol precipitation as in Moore et al.
(2004). PCR was carried out with the universal primers 27F (5’-
\texttt{AGAGTTTGATCMTGGCTCAG}-3’) and 1552R (5’-\texttt{AAGGAGGTGATCCARCCGCA}-
3’) (Lane, 1991; Johnson, 1994) in a PTC 200 DNA Engine Cycler (Biorad,Hercules,
CA) with a thermal profile of 94°C for 3 min and 35 cycles of 94°C for 30 s, 58°C for 30
s and 72°C for 90 s followed by a final extension of 72°C for 5 min. Each 25 µl PCR
reaction mixture consisted of 2 µl of template DNA, 12.5 µl of water, 0.5 µl of both
forward and reverse primers, and 12.5 µl of GoTaq Pre- Mixed Green Master Mix
(Promega Corporation, Madison, WI). Success of PCR amplification was verified via gel
electrophoresis. Sequencing was performed at the Advanced Genetic Technologies Center at University of Kentucky in Lexington, KY using 27F and 1552R primers. Resulting amplicon sequences were trimmed for quality in Sequencher (Gene Codes Corporation, Ann Arbor, MI) using default settings.

Sequences were compared to GenBank with the Basic Local Alignment Search Tool (BLASTN) (Altschul et al. 1997). Multi sequence alignment program MUSCLE (Edgar 2004a; Edgar 2004b) was used to align with sequences from the Genbank database. MEGA (Version 5.2.1) (Tamura et al. 2011) was used to infer phylogenetic relationships. To generate a distance matrix DNADIST under assumptions of Juke and Cantor (1969) and Kimura (1980) was used. Maximum parsimony method with 500 bootstrap replicates was used to generate phylogenetic trees from these distance matrices.

**Statistical Analysis**

One-way ANOVA using JMP statistical software (version 10, SAS Institute Inc., Cary, NC) was carried out, to determine differences in patterns of N utilization by bacteria isolated from 8 different N enrichments; student’s t test was performed for Post-hoc analysis and p values ≤ 0.05 were considered significant. To compare the bacterial growth among 12 different N compounds, a Pearson’s correlation matrix was created using R statistical software (version 2.15.1 for Windows). Mantel test (R statistical software, version 3.0.1 for Mac) was used to evaluate the correlation between phylogenetic distance among bacterial isolates and their growth in various N compounds.

**Results**
Stream physicochemical variables

Physicochemical variables, namely conductivity, DO and turbidity, were significantly different among the three streams (Table 1). Conductivity was lowest in WBM at the time of sample collection as compared to the SS and SYC. Unlike conductivity, DO was highest in WBM and lowest in SS. Turbidity was low in both the forested streams i.e. WBM and SYC, whereas highest values were recorded in the agriculturally impacted SS. However, no significant differences in temperature and pH were observed among streams.

Bacterial isolates

A total of 265 bacterial isolates were obtained from the three streams from the eight different sole-source N compounds. Although no significant differences existed in the total number of bacteria isolated from the 3 different sources (WBM, SS and SYC), there were significant differences in the total number of isolates obtained from each of the 8 different N enrichments (data not shown). A maximum number of bacteria (56 isolates) were isolated from nutrient broth while the enrichment with bacterial protein yielded the least number of bacteria (26 isolates).

Based on 16S rRNA gene partial sequence analysis using BLAST, the majority of the bacterial isolates obtained were highly similar (>97%) to members of the gamma-Proteobacteria (Figure 1). While bacteria similar to beta-Proteobacteria were isolated from all the enrichments, the maximum number of isolates with sequence similarity to this bacterial phylotype was obtained from the nutrient broth enrichment. A small
percentage (3%-12%) of isolates, similar to members of Firmicutes, were obtained from six (ammonium, glycine, tryptophan, urea, bacterial protein and nutrient broth) out of eight enrichments. Only 3% of the isolates obtained from the enrichment with defined mixture of N compounds were closely related to Cytophaga-Flavobacterium-Bacteroides group.

**Bacterial utilization of N compounds**

Heat maps for bacterial growth rate on 12 different N compounds (nitrate, ammonium, urea, glycine, proline, tryptophan, nucleic acid, peptidoglycan, bacterial protein, polyamine, algal exudate and humic matter) were generated based on OD$_{600}$ measurements at different time points.

Distinct differences in bacterial growth rates on N compounds can be deduced from the heat plots (Figure 2). No discernible pattern in growth rate was observed among isolates and irrespective of the enrichment, bacterial isolates had highest growth rate in labile N sources namely nitrate, ammonium, urea, glycine, proline, tryptophan and low growth in nucleic acid and humic matter. All of the isolates obtained from enrichments with tryptophan had similar growth rates in most N sources (Figure 2 D) except for isolate SS86 that high growth rate in only urea, polyamine and algal exudate.

Figure 3 summarizes the patterns of N utilization based on OD$_{600}$ values and displays the percentage of generalists and specialists from each N-enrichment. The majority of the generalists (about 70%) were obtained from nutrient broth and M9 medium with the defined mixture of N compounds while the majority of the specialists
(average=82%) were isolated from M9 medium with nitrate, ammonium, glycine, or bacterial protein as the sole source of N.

Irrespective of the enrichments the bacterial isolates were derived from, there existed varying degrees of correlation in bacterial growth among the 12 different sole-source N compounds (Figure 4). The font size of the correlation values in the matrix is proportional to the strength of the correlation [strong correlation (larger size) and weak correlation (smaller size)]. Bacterial growth on the most simple, readily used forms of N (nitrate, ammonium, urea) were highly correlated with each other; the strongest correlation was between growth in ammonium and urea treatments. The strength of the correlation as depicted in Figure 4 gradually declined when growth in the simplest N compounds was compared to growth in glycine, proline and tryptophan. At the other end of the spectrum, growth on the large organic N compounds was also strongly correlated. Specifically, a strong correlation existed for bacterial growth between nucleic acid, peptidoglycan, bacterial protein, polyamine, algal exudate and humic matter. However there were no significant correlations when bacterial growth was compared between the simple (nitrate, ammonium, urea, glycine, proline and tryptophan) and complex forms (nucleic acid, peptidoglycan, bacterial protein, polyamine, algal exudate and humic matter) of N compounds (correlation values not visible on the plot).

**Phylogenetic trees**

Phylogenetic trees were generated for isolates derived from the eight N enrichments (Figure 5 A-H). Bacteria and respective GenBank accession numbers that had closest
sequence similarity to the isolates obtained are included in the trees (Note: GenBank numbers for the sequences generated in this study will be added after they are obtained). Based on 16S rRNA gene sequence analyses of the isolates, phylogenetic trees revealed no discernible relationship among bacterial taxa pertaining to their pattern of N utilization. For example while bacterial isolate SYC66, from glycine enrichment, with maximum sequence similarity to Comamonas sp (Genebank Acc # HQ200412.1) was categorized as a specialist (Figure 5C), SS56, obtained from nutrient broth, also similar to Comamonas sp (Genebank Acc # HQ200412.1) was characterized as a generalist (Figure 5H). Likewise, most of the isolates with maximum sequence similarity to Pseudomonas sp., from ammonium and bacterial protein, acted as specialists (Figure 5B and G) but isolates from nutrient broth similar to Pseudomonas sp acted as generalists (Figure 5H). This finding was further confirmed by mantel test that revealed absence of any significant correlation (Mantel statistic r: -0.04208 ; Significance: 0.991 ; based on 999 permutations) between phylogenetic distance of the bacterial isolates and their growth rate on different N compounds.

Discussion

Total N pool in streams is composed of a plethora of organic and inorganic forms of N (Wheeler and Kirchman 1986; Zehr and Ward 2002) that vary in their availability to certain species of bacteria (Crawford et al. 1974; Hollibaugh and Azam 1983; Paul et al. 1989; Coffin 1989). In this study we investigated the ability of heterotrophic bacterial isolates to utilize 12 different N compounds and their response were used to broadly categorize the isolates as generalists and specialists. Bacteria isolated from different N
enrichments differed in their patterns of N utilization. However, differences in resource utilization were not related to the taxonomic affinity of the isolates.

Different enrichments yielded isolates with varying breadths of utilization of different forms of N. In addition, across isolates, correlations in growth were observed at the ends of the spectrum of N compounds. Specifically, growth rates on simple N compounds were highly correlated with each other, as was the case for growth on complex organic N compounds. The nature of the N compounds used is varied, as is the mechanism by which N is taken up (Merrick and Edwards 1995). The simple N compounds are labile and readily utilizable (McCarthy and Carpenter, 1983; Wheeler and Kokkinakis 1990; Brookshire et al. 2005) whereas uptake of the more complex compounds requires enzymatic degradation before assimilation (Hoppe 1991; Sinsabaugh et al. 1997). Selection imposed by the enrichments presumably favored isolates that were adept at using particular N in these broad classes (Merrick and Edwards 1995).

In spite of this apparently strong selective pressure, major taxa isolated from the enrichments were similar except for the complex, undefined medium, nutrient broth. The organisms recovered are common in stream ecosystems and were mostly members of the class Gammaproteobacteria (such as Enterobacter Pseudomonas, Serratia, Rahnella Klebseilla etc) that have been reported to be abundant in freshwater habitats (Belt et al. 2007; Das et al. 2007; Newton et al. 2011). Yet, there are other major class of bacteria, namely the Betaproteobacteria and Cytophaga (Manz et al. 1999), which were under-represented in this experiment.
The taxonomic identity of a particular isolate appeared largely unrelated to its pattern of N utilization and whether it is classified as a generalist or specialist. There are three possible explanations. First, perhaps within the range of taxonomic resolution afforded by examination of 16S rRNA genes; there is considerable functional variation among species and subspecies (Philippot et al. 2010). It is often difficult to ascertain the metabolism of organisms based on only 16S rDNA-based phylogenies (Gray and Head 2001; Chesson et al. 2002). 16S rRNA is a widely used tool for bacterial characterization (Woese et al. 1990; Weisburg et al. 1991; Tang et al. 2000) but it is not able to resolve differences among species in the same genus (Fox et al. 1992; Janda and Abbott 2007).

Second, perhaps other properties of the genomes of a given isolate determined functional response and that this other genes were disconnected from the 16S rRNA gene. Similar finding have been reported in a study by Raymond et al. (2002) on photosynthetic bacteria. Consider the classic example of *Escherichia coli*: the same organism can be a symbiont or a pathogen based on a straightforward genetic difference (Kasper et al. 2004). Similarly; a study by Sauer et al. (2002) revealed opportunistic pathogenic nature of *Pseudomonas aeruginosa* that can grow as an harmless symbiont in the host until they reach cell counts sufficient to invade the host’s immune system. Third, potential organisms with similar genomes respond physiologically in different ways based on specific differences in their microenvironments, for example. Prior studies on cyanobacteria (*Synechococcus*) (Ferris et al. 2003) and *Comamonas* species (Ma et al. 2009) have shown that their immediate physiochemical conditions drive evolutionary adaptations for surviving in diverse and complex ecological niches.
In conclusion, bacteria varied in their patterns of growth on various N compounds and strong correlations in bacterial growth were observed at the ends of the spectrum (labile vs recalcitrant) of N compounds. Although enrichments selected for organisms with different N utilization profiles, they were disconnected from their taxonomic identity. The plausible explanation for that being; while adaptation is important for initiating bacterial speciation (Cohan 2001 and 2002), it takes time for mutations to accumulate in neutral markers, such as 16S rRNA, leading to variability in metabolic responses by members of the same genus.
References


Fox GE, Wisotzkey JD, Jurtshuk P Jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. International Journal of


Hutchinson GE. 1959. Homage to Santa-Rosalia or why are there so many kinds of animals. The American Naturalist 93: 145–159.


**Table 1.** Physicochemical variables measured during sediment sample collection from West Branch of the Mahoning (WBM), OH., Sugar Creek near Shirley (SS) and Sycamore Creek (SYC), IN., Values are means and standard errors.

<table>
<thead>
<tr>
<th>Stream</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>Conductivity (µS/cm)</th>
<th>DO (mg/L)</th>
<th>Turbidity (NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBM</td>
<td>12.7±0.04</td>
<td>7.5±0.07</td>
<td>187.4±0.21</td>
<td>8.8±0.31</td>
<td>1.29±0.71</td>
</tr>
<tr>
<td>SS</td>
<td>10.5±0.03</td>
<td>8.1±0.11</td>
<td>365.3±1.02</td>
<td>3.2±0.11</td>
<td>3.42±2.2</td>
</tr>
<tr>
<td>SYC</td>
<td>11.8±0.06</td>
<td>7.8±0.04</td>
<td>351.1±1.03</td>
<td>5.6±0.33</td>
<td>1.88±0.24</td>
</tr>
</tbody>
</table>
Figure 1: Pie charts represented as percentages of major taxonomic groups of bacteria obtained from 8 different sole-source N enrichments.
Figure 2: Heat maps representing patterns of bacterial growth rate on 12 different nitrogen compounds. Each heat map represents the enrichment bacterial isolates were obtained from: A) Nitrate; B) Ammonium; C) Glycine; D) Tryptophan; E) Urea; F) Defined mix of N compounds; G) Bacterial Protein; H) Nutrient Broth. The color legend indicates the optical density.
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Figure 3: Percentage of generalist vs specialist obtained from 8 different sole-source N enrichments.
Figure 4: PEARSON’s correlation matrix to compare bacterial growth among 12 different N compounds. The diagonal of the matrix contain the variables (12 different N compounds used). Left-of-diagonal panels represent correlation plots, whereas the right-of-diagonal panels contain the correlation values for each of those respective plots. Size of the numbers on the right-of-diagonal is proportional to strength of the correlation [strong correlation (larger size) and weak correlation (smaller size)].
Figure 5: Phylogenetic trees based on 16S rRNA gene sequences of bacteria isolated from 8 different N enrichments. Bacteria with closest sequence similarity to the isolates and their respective accession numbers from GeneBank, are included. The trees were generated using the Maximum parsimony method with 500 bootstrap replicates. A) Nitrate; B) Ammonium; C) Glycine; D) Tryptophan; E) Urea; F) Defined mix of N compounds; G) Bacterial Protein; H) Nutrient Broth. The color legend indicates the generalist and specialist.¹

¹Heat plots from Figure 2 will be conjoined with respective phylogenetic tree from Figure 5 after Genebank accession numbers are received and before final thesis submission to Kent State University.
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CHAPTER 3: IMPACTS OF LABILE ORGANIC CARBON CONCENTRATION ON ORGANIC AND INORGANIC NITROGEN UTILIZATION BY STREAM BACTERIA

Abstract

In aquatic ecosystems, C availability strongly influences N dynamics. One manifestation of this linkage is the importance, in the dissolved organic matter (DOM) pool, of dissolved organic nitrogen (DON) that can serve as both a C and a N source, yet, our knowledge about how specific properties of DOM influence N dynamics are limited. To empirically examine the impact of labile DOM on responses of bacteria to DON and DIN, bacterial abundance and community composition were examined in controlled, laboratory microcosms subjected to various dissolved organic carbon (DOC), DON and dissolved inorganic nitrogen (DIN) treatments. Bacterial communities that had colonized glass beads incubated in a stream were treated with varying glucose concentrations and combinations of inorganic and organic N (derived from algal exudate, bacterial protein, and humic matter). Results revealed a strong influence of C availability on bacterial utilization of DON and DIN, with preferential uptake of DON under low C concentrations. Bacterial DON uptake was affected by concentration and by its chemical nature (labile vs. recalcitrant). Labile N sources (algal exudate and bacterial protein) was

1(This chapter has been submitted for publication to the journal Applied and Environmental Microbiology and is under peer review; the author list is as follows: Suchismita Ghosh and Laura G. Leff)
utilized equally well as a N source as DIN, this was not the case for the recalcitrant, humic matter DON treatment. Clear differences in bacterial community composition among treatments were observed based on T-RFLP (terminal restriction fragment length polymorphisms) of 16S rRNA genes. C, DIN, and DON treatments likely drove changes in bacterial community composition that, in turn, affected rates of DON and DIN utilization under various C concentrations.

**Introduction**

Dissolved organic matter (DOM) is the largest component of the organic matter pool in lotic ecosystems (Karlsson et al., 2005; Tank et al., 2010). Concentration and composition of DOM varies spatially and temporally (del Giorgio and Cole, 1998; Church et al., 2000; Kritzberg et al., 2005 and 2006) which, in turn, affects productivity of stream food webs (Richardson, 1991; Wallace et al., 1999; Hassan et al., 2005). Heterotrophic bacteria are major consumers of DOM (Sherr and Sherr, 1996; Azam, 1998) and thus, DOM influences bacterial community composition (i.e., structure) and abundance (Leff and Meyer, 1991; Koetsier et al., 1997; Leff, 2000; Olapade and Leff, 2005).

Processing and fate of organic C is fundamentally and synergistically linked to the N cycle (Redfield, 1958; Turner, 2002; Taylor and Townsend, 2010). Despite this linkage between C and N cycles, many studies focus solely on C-dynamics (Fisher and Likens, 1973; McDowell and Fisher, 1976; Meyer and Tate, 1983) or N-dynamics (Grimm et al., 1981; Triska et al. 1984; Mulholland et al., 2000) rather than their inter-
relationship. In streams, specifically, C and N are tightly linked (Bernhardt and Likens, 2002) and C availability strongly influences N dynamics (Paerl, 1993; Seitzinger, 1994); yet, our knowledge about how specific properties of DOM influence N dynamics is limited.

Nitrogen is an important component of DOM (Wiegner and Seitzinger, 2004) and a major fraction (often more than 50%) of the total dissolved N pool is dissolved organic nitrogen (DON) (Krupka, 1989; Wetzel, 2001; Perakis and Hedin, 2002; Seitzinger and Sanders, 1997). The DON pool is composed of a continuum of compounds ranging from high molecular weight polymers, like polypeptides, to low molecular weight monomers, like amino acids and urea (Antia et al., 1991; Zehr and Ward, 2002). DON also is derived from different sources (e.g., allochthonous versus autochthonous) which influences composition and lability (Seitzinger et al., 2002; Stepanauskas et al., 2000).

Allochthonous sources contribute the majority of refractory DON to streams (Mulholland, 2003), whereas algal derived DON is more labile (Kaplan and Bott, 1982; Mulholland, 1992). DON utilization by heterotrophic bacteria varies; the labile fraction is readily utilized (Iturriaga and Hoppe, 1977; Wolter, 1982; Bell, 1983; Sell and Overbeck, 1992; Brookshire et al., 2005) while the recalcitrant fraction is utilized with the aide of extracellular enzymes (Sinsabaugh et al., 1997).

Reliance on organic versus inorganic forms of N depends on N availability. The ability to take up dissolved inorganic nitrogen (DIN) in the form of nitrate or ammonia is widespread among bacteria; ammonia uptake is energetically favorable but often nitrate is more available (McCarthy and Carpenter, 1983; Wheeler and Kokkinakis, 1990).
Although DON serves as a potential N source (and C) for microbial communities (Antia et al., 1991; Berman and Bronk, 2003; Zehr and Ward, 2002), bacterial metabolism of DON is influenced by inorganic N concentrations (Kaushal and Lewis, 2005). High DIN concentrations inhibit production of enzymes that scavenge N from DON (Chrost, 1991) and reduce extracellular hydrolysis of refractory DON (Chrost, 1991; Munster and de Haan, 1998).

DOM serves as a substrate for bacterial growth (Bott et al., 1984) which in turn increases the demand for assimilation of nitrogen (Bernard and Likens, 2002). DON serves, potentially, as both a C and N source. The ability of DON to meet the metabolic demand for organic C likely influences the assimilative demand for N and whether, energetically, that demand is best met by N from DON or DIN. To empirically examine the impact of labile DOM on responses of bacteria to DON and DIN, bacterial abundance and community composition (based on T-RFLP of 16S rRNA genes, Liu et al., 1997) were examined in controlled, laboratory microcosms subjected to various dissolved organic carbon (DOC), DON and DIN treatments. Bacterial communities that had colonized glass beads incubated in a stream were treated with three labile C (glucose) concentrations and four combinations of inorganic and organic N. Because DON is diverse and compounds vary in bioavailability, three DON sources were examined: humic matter, bacterial protein, and algal exudate. Additional energy expenditure is required for utilization of humic matter and protein because extracellular enzyme production is required for assimilation (Billen, 1991; Chrost, 1990; Hoppe, 1991). In
contrast, algal exudates are typically low molecular weight, more labile, and readily utilized by bacteria (Bronk, 2002; Perez and Sommaruga 2006; Wetzel, 1992).

**Methods**

Bacterial communities used in the experiments were grown on etched soda lime glass beads (4mm in diameter; Fisher Scientific) that were incubated in the West Branch of the Mahoning River, Portage County, Ohio, USA (Olapade and Leff, 2005; Santmire and Leff, 2007). Beads were autoclaved, packed in mesh bags (80 µm mesh size; WildCo- Wild Life Supply, Yulee, FL), and incubated for 45 days in a riffle in the stream.

Water temperature, pH, conductivity, dissolved oxygen (DO) were measured using a Qd/IntelliCAL Rugged Field Kit (Hach Company, Loveland, CO) and turbidity was measured with a Hach turbidimeter, model 2100P when substrates were deployed and retrieved. DOC and total N (TN) concentration were determined using a Shimadzu TOC analyzer (Shimadzu Corporation, Columbia, MD).

Upon retrieval, beads from bags were pooled together and divided into subsamples. A portion of the beads was frozen at -80°C for DNA extraction from the initial bacterial community, another was preserved with 8% paraformaldehyde in phosphate-buffered saline (pH 7.2) for bacterial enumeration, and the remainder was used in experiments as described below.

In the laboratory, each treatment and control was carried out in triplicate. Experiments were performed in flasks containing 10 g of beads in 150 ml of artificial
stream water (ASW; composition per liter: 12 mg NaHCO₃, 7.5 mg CaSO₄ 2H₂O, 7.5 mg MgSO₄, 0.5 mg KCl, 10 mg CaCO₃, 10 mg K₂HPO₄, pH 6.4). Treatments consisted of different nitrogen sources (and concentrations) and different organic carbon concentrations (Table 1). Organic nitrogen sources were bacterial protein, algal exudate, and humic matter (obtained as described below) and inorganic nitrogen was NaNO₃. For each nitrogen treatment, there were three glucose concentrations (low; medium; high). N and DOC concentrations were selected based on average concentrations in the study stream (Olapade and Leff, 2005). Positive controls (without N but amended with low, medium or high concentrations of glucose) were used to examine the effect of glucose amendment on bacterial communities. Negative controls (without C and N amendments) were also performed. Flasks were incubated with shaking (100 rpm) at 25°C and were destructively sampled after 5 or 10 days.

Soluble bacterial proteins were obtained from cultures of Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus aureus incubated at 27 ºC for 24 hours. Proteins were extracted using the Qproteome Bacterial Protein Prep Kit (Qiagen, MD) as per the manufacturer’s protocol and proteins from the three bacteria were pooled. Algal exudates were prepared by growing Chlamydomonas, Chlorella and Synedra (Carolina Biological Supplies, Burlington, NC) in ASW with 20mg/L of NaNO₃. Cultures were grown under constant light for 35 days and processed as described below. Humic matter was derived from senescent red oak (Quercus rubra), witch hazel (Hamamelis virginiana) and corn leaves (Zea mays) extracted overnight, in the dark, in 0.027% NaCl and pooled together. Algae exudates and leaf leachates were filtered through GF/F filters.
(Whatman, Maidstone, UK) and filter sterilized with 0.02 µm Anodisc filters (Whatman, Maidstone, UK). Non-ionic DAX-8 resin (Supelco, Sigma-MO) was used to separate the humic fraction of the leaf leachate from the non-humic fraction. Total DON and DOC concentrations of the bacterial protein, algal exudate and humic matter were measured with the Shimadzu TNM-1 and TOC-5000 analyzer respectively (Shimadzu Corporation, Columbia, MD).

After 5 or 10 days, ASW from the experimental units was filtered through 0.22-µm pore-size polycarbonate filters (Poretics, Livermore, CA) before total dissolved organic carbon (TDOC) and total nitrogen concentration (TN) were determined as above. NO$_3^-$ concentrations were measured via Ion Chromatography (Dionex chromatography system, Thermo Fisher Scientific Inc, CA). pH was measured with a Delta 320 pH meter (Mettler-Toledo, OH).

At each of the two sampling points, 5 g of beads from a given microcosm were preserved in 8% paraformaldehyde for bacterial enumeration. Subsequently, samples were treated with 0.1% tetrasodium pyrophosphate and sonicated at 40 KHz for 5 min (Ultrasonic cleaner, model 2210; Branson Ultrasonics Co., Danbury, CT) to detach bacterial cells (McNamara et al. 2002). Samples were then filtered through 0.2 µm-size black polycarbonate filters (Livermore, CA) and stained with DAPI (4,6-diamidino-2-phenylindole; Porter and Feig, 1980). Bacteria in 10 fields per filter were enumerated via epifluorescence microscopy.

For DNA extraction, 5 g of beads from each microcosm were sonicated as above and bacteria were concentrated by filtration (0.22-µm pore-size polycarbonate filters;
Poretics). Filters were frozen at -80 °C until DNA was extracted using the Power-Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) as per the manufacturer’s instructions with minor modifications as in Feinstein et al., (2009).

PCR of the 16S rRNA genes followed by terminal restriction fragment length polymorphism (T-RFLP) was used to examine bacterial community structure. An equimolar mixture of 5'-ACTCCTACGGAGGCAGCAG-3' (Eub338F-0-III) and 5'-ACACCTACGGGTGAGCAGCAG-3' (Eub338F-I-II), labeled with 6-carboxyfluorescein, and 5'-ACGGGCGGTGTGATTGTA-3' (1392R) (W= A or T; Blackwood et al., 2005) was used. Each 25µl reaction mixture contained GoTaq Flexi DNA polymerase (2.5 U), buffer (1X), MgCl₂ (0.5 mM), bovine serum albumin (0.64 mg ml⁻¹), deoxynucleoside triphosphates (0.2 mM each), forward and reverse primers (0.2 µM each) along with 2 µl of template DNA. Positive controls (Pseudomonas aeruginosa genomic DNA) and negative controls (sterile deionized water) and were run with each set of PCR reactions. Five PCR reactions per sample were carried out in a PTC 200 DNA Engine Cycler (Biorad, Hercules, CA) with a thermal profile of 94°C for 3 min and 40 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 90 s followed by a final extension of 72°C for 7 min. Success of the PCR amplification was verified by pooling the five reactions per sample and performing gel electrophoresis. PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and digested with endonuclease HaeIII (2U) at 37°C for 18 to 24 hours followed by another round of clean up with the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Analysis of PCR products was performed at The Ohio State Plant Microbe Genomics Facility using a 3730 DNA Analyzer (Applied
Biosystems, Foster City, CA). Results were analyzed via Gene Mapper 4.0 (Applied Biosystems, Foster City, CA).

To identify bacterial sequences in microcosms treated with algal exudate and amended with a high glucose concentration, PCR products of 16S rRNA genes were cloned and sequenced. PCR was carried out with the universal primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1552R (5’-AAGGAGGTGATCCARCCGCA-3’) (Lane, 1991; Johnson, 1994) in a PTC 200 DNA Engine Cycler (Biorad, Hercules, CA) with a thermal profile of 94°C for 3 min and 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 90 s followed by a final extension of 72°C for 5 min. Amplified PCR products were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA) before it was ligated into the pGEM-T vector followed by overnight incubation and transformation of competent E. coli cells according to the manufacturer’s protocol (Promega, Madison, WI). Plasmids were isolated from transformants as in Ausubel et al. (1995). Sequencing was performed at the Advanced Genetic Technologies Center at University of Kentucky in Lexington, KY using M13 primers. Plasmid sequence was removed and amplicon sequences trimmed for quality in Sequencher (Gene Codes Corporation, Ann Arbor, MI) using default settings. Sequences were analyzed with BLAST via the National Center for Biotechnology Information (Altschul et al., 1997).

**Statistical Analysis**

Bacterial abundance, DOC, organic N, nitrate concentration and T-RF (terminal restriction fragment) number were compared among treatments via three-way ANOVA
using JMP statistical software (version 10, SAS Institute Inc., Cary, NC) followed by student’s t test and Tukey’s test for Post-hoc analysis, p values ≤ 0.05 were considered significant. To determine the relationship between growth rate and chemical variables (DOC, inorganic and organic nitrogen used), multiple linear regression, with stepwise forward and backward selection, was used. The values to enter and leave the analyses were 0.100 and 0.05, respectively.

The contribution of N treatments, glucose concentrations and the interaction of these factors to variation in TRFLP profiles were determined using redundancy analysis (Blackwood et al., 2003). R statistical software (version 2.15.1 for Windows) was used to determine differences in TRFLP profiles. Variation in relative peak heights (Hellinger distance) and absence and presence of peaks (Jaccard distance) were considered for such analysis. Prior to analysis the TRFLP relative peak heights were square root transformed.

In addition, composition of the bacterial community was examined by calculating the relative abundance of dominant TRF peaks (Leuders et al. 2000; Kitts 2001). Smaller TRF peaks (that contributed less than 2% to the total) in a pattern were lumped together as ‘other TRF’s’. Three-way analysis of variance (ANOVA) followed by Tukey post-hoc tests were used to identify differences in TRF patterns among treatments and times (5 and 10 days); percent relative abundance of TRFs were normalized by log transformation.

**Results**

**Bacterial abundance**
Growth of bacteria was determined based on changes in abundance. For each DON source separately, three-way ANOVAs were used to test for significant differences in bacterial abundance among treatments (Fig. 1). Growth of bacteria in microcosms treated with DON derived from humic matter was relatively modest and, at each of the three glucose concentrations, abundance at the end of the experiment (10 days) was highest in microcosms amended with the highest concentrations of both inorganic and organic N (Fig. 1 A-C). Under high and medium glucose concentrations, growth when inorganic N concentrations were high and organic concentrations low was significantly lower than when DIN and DON were both high revealing bacterial growth on DON. After 5 days of incubation, growth on high inorganic and organic N was more similar to growth on high inorganic and low organic N relative to day 10.

When protein was the DON source, bacterial growth was ten-fold higher than in microcosms with humic matter. However, like humic matter, after 10 days of incubation, bacterial abundance was highest in the high organic and inorganic N treatment under high and low glucose concentrations (Fig. 1 D-F). In contrast, under medium glucose concentration, responses to this treatment were not significantly different from the high inorganic and low organic N treatment. Like humic matter, after 5 days of incubation, bacterial growth under high DIN and high DON was similar to growth in high DIN and low DON, except under high glucose concentration.

Bacterial abundance in microcosms with algal exudate as the DON source was similar to that achieved by the protein amendment. Also, like protein, after 10 days of incubation, highest growth occurred in high DIN and DON treatments (Fig: 1 G- I)
except under medium glucose concentration. After 5 days of incubation, under high and medium glucose concentrations, growth was significantly higher in the high IN and ON treatment, than in the other treatments.

Controls were used to examine effects of glucose concentration alone on bacterial abundance. In these glucose-only controls, cell numbers were significantly lower (10 fold lower after 5 days and 1000 fold lower after 10 days) than in glucose + N amendments (average abundance in glucose only controls= 0.16 - 0.45 [5 days] to 0.07 - 0.36 X 10^6 [10 days]). Negative controls were not amended with DOC or N and cell abundances were low; ranging from 0.9 -1.17 (5 days) to 0.15 - 0.3 x 10^5 cells/ml (10 days).

**DOC utilization**

Average declines in DOC concentration were 67.9% and 83.7% after 5 and 10 days, respectively. Final DOC concentration differed significantly among treatments (data not shown). Irrespective of the DON source and duration of incubation, maximum DOC loss was observed in treatments with low glucose concentration and was lowest in treatments with high glucose, DON and DIN. Also, microcosms with high and medium glucose had greater DOC loss (more bacterial uptake) in treatments with high DON and low DIN compared to treatments with low DON and high DIN.

Because initial differences in DOC concentration impacted final concentrations, DOC loss was expressed as DOC utilization per bacterial cell [loss of DOC (i.e. difference between initial and final DOC concentration) divided by bacterial abundance] and differed significantly among treatments (Fig. 2). Generally, experimental microcosms
with higher bacterial abundance had low per cell DOC use than those with low bacterial abundance; bacterial DOC use per cell tracked patterns of bacterial abundance with some exceptions. At the end of the experiment, for all DON sources under low glucose concentration, bacterial abundance in amendments with high DON and low DIN was similar to that in low DON and high DIN (Fig. 1 A, D, G). However, bacterial DOC use per cell was significantly lower in the low DON and high DIN treatment than the high DON and low DIN treatment (Fig. 2 A, D, G).

Since bacterial DOC use per cell was a function of abundance, in positive controls, DOC use per cell (average= 84.35 pg/ cell ± 9) was higher than DOC use in experimental microcosms. DOC was not added to the negative controls, thus DOC use per cell was below detection.

N Utilization

Bacterial utilization of organic and inorganic N (based on declines in DON and DIN in microcosms) differed significantly among treatments (Fig. 3). Irrespective of the DON source, under high DON and high DIN and low glucose concentration conditions, bacterial use of organic N was significantly higher than inorganic N after both 5 and 10 days. (Fig. 3: A, D, G). However, bacteria utilized organic and inorganic nitrogen equally well under medium and high glucose concentrations when bacterial protein and algal exudate was the DON source (Fig. 3 E- F; H - I). In microcosms treated with humic matter, under both medium and high glucose concentration, bacterial use of DON was lower than DIN in amendments with high DON and high DIN (Fig. 3 B, C).
To determine if changes in pH played a role in bacterial responses, pH was measured initially and after 5 and 10 days. Initial pH values ranged from 6.50 – 7.45 and no significant differences in pH was observed; final values ranged from 6.25-7.20 (5 days) to 6.05 – 6.79 (10 days).

TRFLP

Based on redundancy analysis (RDA) of 16S rRNA gene T-RFLP, bacterial community structure differed significantly among treatments for all DON sources (p <0.05). The largest percentage of variation in community structure was explained by the interaction between N-treatment and glucose concentration (Table 2). Generally, the percent of variation explained by this interaction was higher after 5 days (average =83%) than after 10 days (average= 60%).

For bacterial communities in humic matter treatments, composition varied among glucose concentrations and N treatments after 5 days (Fig. 4 A). Yet, after 10 days, only bacterial communities under high glucose and low DIN and DON concentrations were spatially separated on RDA axis 1, whereas, communities from other treatments were clustered relatively closely on this axis (Fig 4 B). Other treatments varied along RDA axis 2 but, generally, those with the same glucose concentrations were the most similar.

For the humic matter amendments, number of T-RFs were highest in the treatment with low DON and high DIN under high glucose concentration but did not differ significantly among the rest of the treatments after 5 days (Fig.5 A). However, after 10 days there were significant differences in the number of T-RFs among treatments with
highest numbers in microcosms treated with high DON and DIN under low glucose concentration (Fig. 5 B).

There were also significant differences in relative abundance of TRFs (Fig. 6 A-B). For example; after 5 days, under low and medium glucose concentrations TRF -2 and TRF-4 had low relative abundance but were more dominant after 10 days. TRF-3 had high relative abundance in all treatments except under high DON, low DIN and low glucose. TRF-12 and TRF-19 was absent in all treatments after 5 days but together contributed 60% of the community in the treatment with low DON and high DIN under high glucose concentration after 10 days.

When bacterial protein was the DON source, after 5 days, bacterial community structure differed among glucose and N treatments with no discernible pattern based on treatment (Fig 4 C). Yet, after 10 days, communities became more similar with varying degrees of overlap. Under high glucose concentration, two N-treatments (low DON + high DIN and low DON + low DIN) had distinct communities (Fig 4 D). Number of T-RFS differed significantly among treatments after 5 days with maximum numbers under high glucose concentrations (Fig. 5 C). However, after 10 days the number of T-RFS decreased in all treatments and there were few significant differences in number of peaks (Fig. 5 D).

Significant differences among treatments were also observed in relative abundance of TRFs (Fig. 6 C-D). Bacterial communities became more uniform in all the treatments as they were dominated mostly by TRF-2, 6 and 7 after 10 days.
Composition of bacterial communities treated with algal exudate differed significantly among treatments after 5 days; high glucose concentration with high DON and low DIN as well as low DON and high DIN conditions had the most distinct communities (Fig 4 E). However, after 10 days, communities in the high glucose treatments clustered together regardless of N treatment; this was also somewhat the case for the medium and low glucose concentrations (Fig 4 F). As observed in the humic matter amendments, the total number of TRFs did not differ significantly between 5 and 10 days. Irrespective of incubation period, no significant differences in number of peaks were observed among treatments except for: low glucose, high DON and low DIN treatment after 5 days and high glucose, high DON and high DIN treatment after 10 days which had relatively low number of peaks (Fig. 5 E-F). Relative abundance of specific TRFs differed significantly among treatments (Fig. 6 E-F). TRF-1 was dominant in all treatments after 5 days. It continued to be the dominant TRF at the end of 10 days in treatments with low and medium glucose amendment but was absent in high glucose concentration. TRF-2 was absent in all treatments with low glucose amendments after 5 days. However, it showed a striking increase (accounting for 72%-88% of the community) in treatments with high glucose after 10 days. Cloning and 16S rRNA sequencing followed by BLAST comparisons to GenBank performed on samples from this treatment revealed that 75% of the clones had a high degree of sequence similarity to Pseudomonas spp. while, the other 25% had more similarity with members of the Enterobacteriaceae (Serratia spp, Klebseilla spp. and Proteus spp. as nearest neighbors).
(Table 3). Out of 12 clones, seven demonstrated maximum sequence similarity (95-97%) to strains of *Pseudomonas aeruginosa*.

**Discussion**

Carbon availability exerts strong controls of bacterial N dynamics (Seitzinger, 1994; Jones et al., 1995; Currie, 1999). In aquatic ecosystems, heterotrophic bacteria meet their N demand via utilization of inorganic and organic N, but the interplay between DIN, DON, and labile DOC are not well understood. In this study, we manipulated the supply of labile C to the bacterial community and then followed loss of N, C, and shifts in bacterial community abundance and composition. We found that the C supply impacted bacterial N utilization, and reliance on organic versus inorganic N was determined by changes in labile C concentration and quality of organic N.

In freshwater, more than 50% of total dissolved N is DON (Krupka, 1989; Chapman et al., 1998; Wetzel, 2001; Willett et al., 2004) which varies in composition and bioavailability (Berman and Bronk, 2003). DON is an important N source for heterotrophic bacteria (Wheeler and Kirchman, 1986; Hoch and Kirchman, 1995; Middelboe et al., 1995; Rosenstock and Simon, 2001). Since DON-derived N can be either used directly (Berg et al., 1997; Mulholland et al., 2002) or indirectly via extracellular enzymes (Palenik and Morel, 1990; Berg et al., 2002; Mulholland et al., 2003b) bacterial uptake depends on the nature of the DON pool. In our study, different DON sources elicited different bacterial responses. For example, bacteria preferentially used DIN over humic-DON under medium and high glucose concentrations since humic
derived DON is more resistant to biological degradation (Wetzel, 1992) and more energy is required (Sinsabaugh et al., 1997). However, bacterial uptake of humic-DON was higher than DIN under low glucose concentration suggesting that the humic compounds served as sources of both N and C (Carlsson et al., 1993) under carbon limiting conditions. The refractory nature of humic compounds accounts for the low bacterial abundance in microcosms treated with humic matter. Like humic DON, algal derived DON and bacterial protein were used more than DIN under low glucose concentrations and were more labile (Bronk, 2002) yielding greater bacterial abundances. In contrast, under medium and high glucose concentrations DIN and DON were used equally suggesting that even under conditions where C is less limited these DON mixtures are useful as C and N sources.

In addition to impacts caused by differences in the type of DON, the impacts of addition of labile DOC were also examined. DOC is an important driver of bacterial abundance (Pomeroy and Wiebe, 2001) and, as expected, bacterial growth was stimulated by the addition of labile DOC (Eiler et al., 2003). In microcosms with high bacterial abundance, rapid decline in DOC from the water column along with low DOC use per cell suggests there was the potential for strong competition for carbon within the bacterial community (Hibbing 2010).

Heterotrophic bacteria differ in their utilization of C and N based on their metabolic capabilities and thus bacterial community composition plays a critical role in nutrient uptake (Reed and Martiny 2007). Bacterial community structure differed among DON sources and treatments. Previous research reveals that while differences in bacterial
community composition can influence metabolism of organic N (Guldberg et al. 2002; Findlay 2003), in freshwater ecosystems, changes in resources (nutrient concentration and quality) often trigger responses in community metabolism (Vrede 2005). Therefore, community composition may be both a driver of differences in C and N utilization and an attribute that is influenced by C and N treatments.

Variation in community metabolism can result from physiological acclimation, changes in community composition, or a combination of both (Fisher et al. 2000; Eiler et al. 2003; del Giorgio and Gasol 2008; Allison and Martiny 2008). In the experimental microcosms, differences in utilization of N under varying C concentrations is possibly due to shifts in abundance of bacteria with different nutritional needs resulting in changes in community composition. Community composition and functional response are highly correlated (Comte and del Giorgio 2010) and shifts in community composition can be accompanied by changes in the hydrolytic ectoenzyme activity (Pinhassi et al. 1997).

We observed maximum differences in community structure among treatments after 5 days, which diminished after 10 days when bacterial protein and algal exudate were the DON source. Perhaps as the resources were depleted, lower bioavailability of C and N led to slow growing, stable communities which are dominated by fewer TRFs with similar capabilities to utilize the left over resources (Hibbing et al. 2010). However, this was not observed in microcosms treated with humic matter. In this case, lower bioavailability of recalcitrant compounds may lead to more diverse communities composed of different TRFs because enzymatic breakdown of complex compounds by some members allows for uptake of the products by others in the community (Morris and
Rouse 1985; Sinsabaugh et al., 1997). This possibility is supported by the more balanced contributions of multiple T-RFs in the communities of humic treatments relative to the other DON sources. Competition may also contribute to these differences in bacterial community composition among treatments as bacteria interact to obtain the C and N required for their growth (Hibbing et al. 2010).

The shift to dominance by a limited subset of TRFs was most obvious in microcosms treated with high concentrations of glucose with algal exudate as the DON source. Regardless of N treatment, one TRF accounted for approximately 80% of the community. Based on 16S rRNA gene sequencing, these dominant organisms were *Pseudomonas*, a genus that is widely distributed in aquatic ecosystems (Pellett et al. 1983; Romling et al. 1994). Being genetically and metabolically versatile, *Pseudomonas* is ubiquitous and occupy multiple ecological niches (Bergan 1981). Several sequences had strong similarity to *Pseudomonas aeruginosa*, which is an efficient competitor for resources; they produce antibiotics (Hibbing 2010) and deploy toxins to attack cell walls of other bacterial competitors in a community (Russell 2011). In our experimental microcosms treated with algal exudate and amended with high glucose, *Pseudomonas sp.* possibly scavenged C and N, limiting their acquisition by other members of the bacterial community.

Overall, there was a strong linkage between C availability and bacterial utilization of DON over DIN. Bacterial utilization of DON was not only related to availability of inorganic N but also to the nature of the DON (labile versus recalcitrant). Difference in use of organic N were strongly associated with differences in community composition.
Likely, C, DIN, and DON treatments drove changes in bacterial community composition that, in turn, affected rates of DON and DIN utilization in response to various C concentrations.
References


Table 1. Carbon and nitrogen treatments used in the experiment. Organic nitrogen sources consisted of bacterial protein, algal exudate and humic matter, and nitrate was the source of inorganic nitrogen. Three concentrations of organic carbon, in the form of glucose, were used for each nitrogen treatment. OH - Organic nitrogen high concentration, OL – Organic nitrogen low concentration, IH - Inorganic nitrogen high concentration, IL – Inorganic nitrogen low concentration.

<table>
<thead>
<tr>
<th>Carbon Treatment</th>
<th>Nitrogen treatments</th>
<th>Glucose (mgC/L)</th>
<th>Organic Nitrogen (mgN/L)</th>
<th>Inorganic Nitrogen (mgN/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Low</td>
<td>OH+IL</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C-Medium</td>
<td>OH+IL</td>
<td>10</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>10</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>10</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C-High</td>
<td>OH+IL</td>
<td>25</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>25</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>25</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2. Percentage variation in bacterial community structure, after 5 and 10 days, explained by N treatments, glucose concentration and the interaction between N- treatments and glucose concentration, from redundancy analysis of 16S rRNA gene TRFLP profiles.

<table>
<thead>
<tr>
<th>DON source</th>
<th>Variation explained by N-treatment</th>
<th>Variation explained by glucose concentration</th>
<th>Variation explained by N-treatment * glucose concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
<td>10 days</td>
<td>5 days</td>
</tr>
<tr>
<td>Humic matter</td>
<td>11%</td>
<td>15%</td>
<td>14%</td>
</tr>
<tr>
<td>Bacterial protein</td>
<td>12%</td>
<td>3.6%</td>
<td>17%</td>
</tr>
<tr>
<td>Algal exudate</td>
<td>13%</td>
<td>8.1%</td>
<td>20%</td>
</tr>
</tbody>
</table>
Table 3. Best matches of the different sequences obtained from the microcosms treated with Algal exudate and amended with high glucose, DON and DIN.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Length of sequence (bp)</th>
<th>Gene Bank Accession</th>
<th>Best match (BLASTN)</th>
<th>% Identity/E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLAEhc1</td>
<td>1200</td>
<td>KC211291.1</td>
<td><em>Pseudomonas aeruginosa</em> strain KLU02</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc2</td>
<td>1029</td>
<td>JQ659882.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>96/0.0</td>
</tr>
<tr>
<td>CLAEhc3</td>
<td>1046</td>
<td>JQ659882.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc4</td>
<td>1241</td>
<td>JQ659882.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>96/0.0</td>
</tr>
<tr>
<td>CLAEhc5</td>
<td>649</td>
<td>JF708077.1</td>
<td><em>Pseudomonas aeruginosa</em> strain mpc1</td>
<td>95/0.0</td>
</tr>
<tr>
<td>CLAEhc6</td>
<td>641</td>
<td>KC503912.1</td>
<td><em>Pseudomonas</em> sp. ZS-1</td>
<td>77/2e-10</td>
</tr>
<tr>
<td>CLAEhc7</td>
<td>694</td>
<td>JN622013.1</td>
<td><em>Pseudomonas</em> sp. WC-1</td>
<td>88/2e-22</td>
</tr>
<tr>
<td>CLAEhc8</td>
<td>1230</td>
<td>KC503912.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc9</td>
<td>1193</td>
<td>KC211291.1</td>
<td><em>Pseudomonas aeruginosa</em> strain KLU02</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc10</td>
<td>378</td>
<td>GU569122.1</td>
<td>Uncultured <em>Rahnella</em> sp. clone JBXB28</td>
<td>80/9e-47</td>
</tr>
<tr>
<td>CLAEhc11</td>
<td>1292</td>
<td>HQ018868.1</td>
<td><em>Klebsiella</em> sp. GX17</td>
<td>92/0.0</td>
</tr>
<tr>
<td>CLAEhc12</td>
<td>1300</td>
<td>NR_041979.1</td>
<td><em>Serratia ficaria</em> strain DSM 4569</td>
<td>92/0.0</td>
</tr>
</tbody>
</table>
Figure 1: Increase in bacterial abundance after 5 and 10 days of incubation; A-C: humic matter, D – F bacterial protein, G- I algal exudate as DON source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L=low concentration. Values are means and standard errors. Different letters above bars indicate significant differences at p <0.05.
Figure 2: Utilization of dissolved organic carbon (DOC) per bacterial cell in picograms (pg), after 5 and 10 days of incubation; A-C: humic matter, D-F bacterial protein, G-I algal exudate as DON source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, L=inorganic N and H=high concentration, L= low concentration. Values are means and standard errors. Different letters above bars indicate significant differences at p <0.05.
Figure 3: Utilization of organic and inorganic nitrogen per bacterial cell in picograms (pg), after 5 and 10 days of incubation. A-C: humic matter, D-F bacterial protein, G-I algal exudate as DON source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L= low concentration. Values are means and standard errors. Different letters above bars indicate significant differences at p <0.05.
Figure 4: Ordination plots from redundancy analysis of 16S rRNA gene TRFLP peak relative abundance after 5 (A, C, E) and 10 days (B, D, F). A and B= humic matter, C and D=bacterial protein, E and F=algal exudate. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L= low concentration. Values are means and standard errors.
Figure 5: Number of peaks of 16S rRNA gene TRFLP profiles, after 5 and 10 days of incubation. Graph A) after 5 days and B) after 10 days with humic matter as the organic nitrogen source; C) after 5 days and D) after 10 days with bacterial protein as the organic nitrogen source; E) after 5 days and F) after 10 days with algal exudate as the organic nitrogen source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L=low concentration. Values are means and standard errors. Different letters above bars indicate significant differences at p <0.05.
Figure 6: Percentage dominant TRF’s, after 5 and 10 days of incubation. Graph A) after 5 days and B) after 10 days with humic matter as the organic nitrogen source; C) after 5 days and D) after 10 days with bacterial protein as the organic nitrogen source; E) after 5 days and F) after 10 days with algal exudate as the organic nitrogen source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L=low concentration.
CHAPTER 4: RESPONSE OF SEDIMENT BACTERIAL COMMUNITIES TO TEMPORAL VARIATION IN THE DISSOLVED ORGANIC MATTER POOL OF AN AGRICULTURALLY IMPACTED STREAM

Abstract

Bacterial metabolic pathways that drive biogeochemical reactions are catalyzed by microbial extracellular enzymes, which are likely to be linked to constituting taxa in a given microbial community. Therefore, it is important to focus on the effects of altered community structure on ecosystem functions such as activity of enzymes important in biogeochemical cycling of important elements such as C and N. However, weak coupling between structure and function of natural microbial communities, calls for examining their links as a result temporal alterations in stream DOM pool. In this study, we determined temporal patterns of bacterial community structure and potential microbial enzyme activities in response to seasonal changes in stream DOM pool. Water and sediment samples were collected on 6 different dates from an agriculturally impacted stream in Indiana, with disrupted flow regimes. TRFLP analysis of 16S rRNA genes was used to determine changes in bacterial communities structure, and substrate analogues linked to fluorescent molecules were used to study 5 different extracellular enzymes as measures of ecosystem functions. Results suggest temporal changes were pronounced

1(This chapter will be submitted to the journal Environmental Microbiology and the author list is as follows: Suchismita Ghosh and Laura G. Leff)
and remarkably variable among the enzymes tested. Differences in enzyme activities were highly correlated to DOM composition, N availability and discharge. Bacterial community structure also differed significantly over time and was driven by seasonal changes in stream environmental conditions. However, the absence of link between bacterial community structure and potential enzyme activity is indicative of functional redundancy in microbial communities.

Introduction

Streams are spatially and temporally heterogeneous environments with patches of varied metabolic activities (Larned et al. 2010; Winemiller et al. 2010). Temporal variation in environmental conditions (such as temperature, nutrients, and organic carbon) drives differences in microbial activity over time (Jones and Lock, 1993; Wilczek et al., 2005; Sinsabaugh and Shah, 2010, 2011). Insights into relationships between community structure and function can be gained by simultaneous analyses of alteration in bacterial community composition and bacterial mediated processes across spatial or temporal gradients of environmental conditions (Findlay et al., 2003; Docherty et al., 2006; Findlay and Sinsabaugh, 2006; Comte and del Giorgio, 2009).

Bacterial community structure and function may appear coupled or uncoupled based on ecosystem properties, targeted attributes, and methods used (Langenheder et al., 2005; Langenheder et al., 2006; Wertz et al., 2006; Östman et al., 2010). In some cases, the relationships between structure and function are examined simultaneously across a temporal or spatial gradient of environmental conditions (Loreau et al. 2001; Hooper et
Possible outcomes of such studies include alteration in functional response without any change in bacterial community structure. Another possibility is that neither community structure nor function responds to environmental differences because functional redundancy or compensatory effects leave the functional activity unaltered (Kritzberg et al., 2006; Allison and Martiny, 2008). Lastly, both structure and function maybe altered in similar ways by environmental conditions suggest that these attributes are coupled.

Dissolved organic matter (DOM) is the most abundant form of organic matter in running waters (Karlsson et al., 2005; Tank et al., 2010) and plays a central role in meeting carbon and energy demands of stream heterotrophic bacteria. The DOM pool is composed of an array of compounds from different sources (i.e., allochthonous versus autochthonous) with considerable chemical heterogeneity within the broad categories of ‘labile’ and ‘recalcitrant’ fractions. Responses of bacterial communities in streams to the DOM pool depend on quality, quantity, and source (Koetsier et al., 1997; Leff, 2000; Leff and Meyer, 1991).

Bioavailability or quality of DOM varies among sources and, typically, autochthonous sources (such as algal exudates) yield more labile DOM (Wolter 1982; Moran 1990; Sell and Overbeck 1992). In contrast, allochthonous sources (such as compounds resulting from decomposition of litter of terrestrial plants) are generally perceived as being more recalcitrant (Moran 1990; Kritzberg et al. 2004). Yet, bacteria have the ability to utilize these recalcitrant compounds, particularly through production of
extracellular enzymes that aid in degradation of complex compounds into readily assimilated forms (Hoppe 1991; Meyer-Reil 1991; Sinsabaugh 1994).

Seasonally, the DOM pool changes in streams and is largely driven by catchment characteristics (Westerhoff and Anning 2000) and properties of the DOM sources (for example, input of leaves in autumn in forested temperate regions). While DOM composition is largely affected by land use (Oni et al. 2011; Williams et al. 2010) and soil type (Fellman et al. 2009; D‘Amore et al. 2010), concentration and bioavailability are influenced by catchment hydromorphology (Mullholland 2003; Aitkenhead-Peterson et al. 2003). Seasonal changes in biotic demand (Fenner et al. 2005) and dominant hydrologic flow paths (Schiff et al. 1997) drive the extent to which DOM is altered as it passes through the watershed. The composition and biodegradability of DOM in streams varies with source material and extent of biological transformations (Kaplan and Bott 1983).

Bacterial communities use various extracellular enzymes for DOM utilization (Amon and Berner 1994; Baltar et al. 2010). Since extracellular enzymes catalyze a rate limiting step in DOM processing, factors that affect their production, availability and activity have a strong impact on bacterial DOM uptake (Arnosti 2003). The synthesis and activity of these enzymes are regulated by complex, multistep, induction/repression mechanisms inside the bacterial cell (Siuda 1984; Hoppe et al. 1988). Also, various environmental factors (such as temperature, pH, substrate concentration and composition etc.) influence extracellular enzyme activity (Amon and Berner 1994; Chróst and Siuda 2002). At the ecosystem level, bacterial enzyme production is influenced by
environmental factors; while at the micro-habitat scale, it is regulated by enzyme-substrate interactions, such as inhibition, adsorption, stabilization and humification (Sinsabaugh et al. 1991).

Seasonal changes in the DOM pool coupled with the importance of DOM to bacteria suggest that temporal changes in the structure and function of the bacterial community will be driven, in part, by change in the relative importance of recalcitrant versus labile compounds. Also, fluctuations in hydrologic regime influence availability of certain nutrients (Pinay et al. 2007) to microbial communities and along with altering physicochemical conditions. For example, N concentrations in streams can change due to variation in hydrologic and moisture regimes (drying and flooding), (Sahrawat et al. 1980, Poff and Ward 1989).

To investigate the effects of temporal variation in DOM on bacterial community structure and function, water and sediment samples were collected on six dates from an agriculturally impacted stream in Indiana. This stream experiences seasonal changes in the DOM pool attributable to spikes in autochthonous production in spring when algal biomass dramatically increases as nutrients from fertilizer cause high concentrations of nitrate (Lathrop 2006; Baker et al. 2006; Baxter et al. 2012). However the algal growth decreases over time with both decline in N input and discharge. Function of the microbial community in the stream sediment, was assessed by examining activity of five extracellular enzymes and bacterial community composition (structure) was examined based on T-RFLP of the 16S rRNA genes (Blackwood et al. 2003).
**Methods**

Samples were collected from an agriculturally impacted, tile-drain fed stream in Indiana, Leary Weber Ditch (LWD). LWD is a tributary of Sugar Creek in Hancock County and approximately 75% of the basin’s land use is agriculture (Baker et al. 2004; Lathrop 2006; Stone and Wilson 2006). During summer, flow is disrupted when input from tile drains declines leaving disconnected stream reaches with standing water (Lathrop 2006).

Samples were collected over the course of the annual hydrologic cycle of the stream in April, June, August, September, November and December 2011. Water and sediment samples were collected from the center, right and left bank of the stream at each of the 5 sites (about 25 m apart) along the length of the stream and samples from all the 5 sites were pooled to obtain a representative, composite sample. Three such composite water and sediment samples were collected on each date. Environmental parameters namely, water temperature, pH, conductivity, dissolved oxygen (VWR Symphony meter, model- 11388-314) and turbidity (Hach turbidimeter, model 2100P), were measured in the field, on each date.

Sediment from the composite samples was sub-sampled; one fraction was frozen at -80°C for DNA extraction and enzyme analysis, another was preserved at 4°C in 4% paraformaldehyde for bacterial enumeration and the remainder was dried at 60°C for 24 hours to determine percent moisture and subjected to combustion at 500°C for five hours to determine organic matter content. Water samples were used for determination of DOC, nitrate concentration, DOM composition and chlorophyll *a* (chl *a*) concentration.
To measure DOC and N concentrations, water samples were filtered through GF/F filters (Whatman, Maidstone, UK) and subsequently through 0.22 µm filters (Whatman, Maidstone, UK). The filtrate was acidified with HCl and total DOC and N concentrations were determined using a Shimadzu TOC5000 analyzer with a TNM-1 (Shimadzu Corporation, Columbia, MD). Nitrate (NO$_3^-$) concentrations were measured via Ion Chromatography (Dionex chromatography system, Thermo Fisher Scientific Inc, CA).

DOM was fractionated as in Wallace et al. (2008) into humic and non-humic fractions; the latter was further divided into basic (includes amino sugars, amino acids, proteins, aromatic amines) and acidic (includes hydroxyl acids, sugar acids, fatty acids,) fractions. Separation of humic from non-humic fractions was carried out using non-ionic DAX-8 resin (Supelco, Sigma-Aldrich, MO). Non-humic fractions were then subjected to cation exchange resin IR120H (hydrogen form, Sigma-Aldrich, St. Louis, MO) to separate the basic fraction and IRA402 anion exchange resin (chloride form, Fluka, Sigma-Aldrich, St. Louis, MO) to separate the acidic fraction. DOC concentrations from each fraction, as well as the resin bleed, were measured with the Shimadzu TOC5000 analyzer (Shimadzu Corporation, Columbia, MD).

Chl a concentrations were determined as in Mulholland et al. (2004). Water samples were filtered through GF/F filters (0.7- m nominal pore diameter, Whatman) and frozen at -20°C for later analysis. To extract chl a, filters were submersed in 95% ethanol, heated at 79°C for 5 minutes, and then incubated in the dark for 24 hours at room temperature. Absorbance was measured using DU 730 Beckman coulter UV/Vis
spectrophotometer (Beckman Coulter Inc., CA) at 664 and 750 nanometers (nm), followed by acidification of the samples with 0.1 N HCl and re-measurement of absorbance. Benthic chla was extracted by treating 2 g of sediment with 95% ethanol in the dark for 24 hours at room temperature and concentration was determined as above.

To enumerate bacteria, paraformaldehyde preserved samples were treated with 0.1% tetrasodium pyrophosphate and sonicated at 40 KHz for 5 min in a Branson 2210 ultrasonic bath (Branson Ultrasonics Co., Danbury, CT) (McNamara et al. 2002) to detach cells from sediment particles. Samples were filtered through 0.2 μm pore size black polycarbonate filters (Poretics, Livermore, CA) and stained with 4’6- diamidino-2-phenylindole (DAPI) (Kepner and Pratt 1994). Bacteria in fifteen fields were counted for each sample using epifluorescence microscopy.

Function of the sediment microbial community was assessed by measuring activities of α-glucosidase (AG), β-glucosidase (BG), N-acetylglucosaminidase (NAG), leucineaminopeptidase (LAP) and laccase (LAC). For each sample, 1.0 g (dry mass equivalent) of sediment was homogenized with a 2-speed hand blender (Hamilton Beach, Minneapolis, MN) in 125 ml sodium acetate buffer (pH = 5.0) and the resulting slurry was loaded into 96-well black plates with negative control and quench wells as in Saiya-Cork et al. (2002). Fluorescent 4-methylumbelliferone (MUB)-linked substrates (Sigma-Aldrich, St. Louis, MO) were used for AG, BG, NAG and LAP, assays as in Sinsabaugh et al. (1997). NAG assays were incubated for 30 min whereas other enzymes were incubated for 2 hours at 20°C. After incubation, enzyme activity was halted with 10 μL 0.5 N NaOH. A synergy HT Microplate Reader (BioTek, Winooski, VT) was used to
measure fluorescence at 365 nm excitation and 450 nm emission. LAC enzyme activity was estimated as in Courty et al. (2005) with minor modifications. Sediment samples were homogenized in buffer as described above, but loaded into clear 96-well plastic plates, with 50 μL of 2 mM 2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS: Sigma-Aldrich, St. Louis, MO) and incubated for 1 hour at 20°C. Oxidation of ABTS was estimated by measuring absorbance at a wavelength of 420 nm.

Bacterial community structure was assessed by terminal restriction fragment length polymorphism (TRFLP) of 16S rRNA genes. Power-Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) was used to extract DNA from sediments, as per manufacturer’s protocol with minor modifications as in Feinstein et al. (2009). PCR was performed by using an equimolar mixture of 5′-ACCTCTACGGGAGGCWGC-3′ (Eub338F-0-III; labeled with 6- carboxyfluorescein) and 5′-ACACCTACGGGTGGCWGC-3′ (Eub338F-I-II; labeled with 6- carboxyfluorescein) as forward primers and 5′-ACGGGCGGTGTGTACA-3′ (1392R) as reverse primer (W= A or T; Blackwood et al., 2005). GoTaq Flexi DNA polymerase (2.5 U), buffer (1X), MgCl₂ (0.5 mM), bovine serum albumin (0.64 mg ml⁻¹), dNTPs (0.2 mM each), forward and reverse primers (0.2 μM each) along with template DNA was present in a 50μl reaction mixture. PCR was carried out in PTC 200 DNA Engine Cycler (Biorad, Hercules, CA) for 35 cycles with a thermal profile of 94°C for 3 min and 30 cycles of 94°C (30 s), 57°C (30 s), and 72°C (1 min 30 s) followed by a final extension of 72°C for 7 min. PCR reactions were carried out in triplicate (and then pooled) and the success of
amplification was verified by gel electrophoresis with 1% agarose gel in 1X Tris-borate EDTA buffer.

PCR products were digested overnight with Hae-III at 37°C (2U, New England Biolabs, Ipswich, MA) and purified with Qiaquick PCR purification kit (Qiagen, Valencia, CA). Digested PCR products were sent to the Ohio State Plant Microbe Genomics Facility. T-RFLP analysis was performed using Applied Biosystems 3730 DNA Analyzer and the results analyzed via GeneMapper 4.0 (Applied Biosystems, Foster City, CA). Peaks between 50 and 600 bp were included in the analysis if they represented ≥0.5% of the total relative peak height for a given sample.

Statistical analysis:

One-way ANOVA followed by post hoc Tukey HSD (performed using JMP [version 10, SAS Institute Inc., Cary, NC]) was used to determine differences in physicochemical variables (i.e. water temperature, pH, conductivity, dissolved oxygen (DO), turbidity, stream discharge, DOC and TN) and biological variables (bacterial abundance and enzyme activity) among dates. Values p < 0.05 were considered significant.

Multiple linear regression, with stepwise forward and backward selection, was used to determine the relationship between bacterial variables (bacterial abundance, and enzyme activity) and physicochemical factors (concentration of chla, DOC fractions, TN concentrations and stream discharge). The values to enter and leave the analyses were 0.25 and 0.10, respectively. A minimum Akaike Information Criterion (AIC) [Akaike(1973,1987); Bozdogan (1987, 2000)] strategy was used to select between the
competing multiple regression models and the “best” subset of predictors (environmental variables). The model with the minimum value of AIC was considered the “best” approximation of the true model for each subset of predictors.

Redundancy analysis (RDA) was performed to determine the potential physiochemical parameters responsible for the variation among T-RFLP profiles as in Blackwood et al. (2003). To examine differences between profiles, analyses were performed using R statistical software (ver. 2.13.2 for Windows). Relative peak heights (Hellinger distance) as well as peak presence or absence (Jaccard distance) were considered for the analyses. TRFLP relative peak heights were square root transformed prior to analysis. Results were visualized as 2-dimensional ordination plot, which showed the distribution of samples along the first and second RDA axis.

Results

Abiotic variables

Over the course of the study, physicochemical variables varied among dates (Table 1). Water temperature differed significantly among dates and displayed a typical seasonal pattern with lowest temperatures in December and highest temperatures in August and September. Unlike temperature, pH did not change over time and remained near neutral ranging from 7.0 and 7.7. Conductivity differed significantly among dates; highest values were in November and December while lowest were in September. Like conductivity, turbidity also varied significantly among dates. Turbidity was lowest in April but increased over time with highest values (2.5-fold increase) in November. DO
was lowest in August and September but was not significantly different among the other four dates (April, June, November and December).

Discharge in LWD was variable throughout the year and was significantly different among dates (Fig. 1). Highest discharge occurred in April and discharge decreased over time with lowest discharge in August. There was no flow in September when LWD consisted of disconnected stream reaches that had standing water. Flow resumed in November after rain events but remained low through December.

Total dissolved nitrogen concentration also differed significantly over time, and was generally higher in April and June compared to the other dates (Fig. 2). Nitrate concentration showed a similar pattern with highest concentrations in June (7.5 mg/L). A sharp decline in nitrate concentration (<1.7 mg/L) was observed in September. Overall, nitrate concentrations were significantly higher in summer than in fall.

DOC concentrations in water samples were highest in June and decreased over time with lowest concentrations in December (Fig. 3). Similarly, the labile fraction (both cationic and anionic) displayed a seasonal pattern; with maximum concentrations of labile DOC in June that declined by approximately 1.8-fold in December. Unlike the labile fraction, DOC concentration of the humic fraction did not differ significantly among dates. However, the contribution of humics to the total DOC concentration did differ change over time. The humic fraction contributed only 33% of the total DOC concentration in June while in December it accounted for about 68% of the total DOC concentration.
Biotic variables

Chla concentration in water and benthic samples varied with a discernible seasonal pattern; June had highest concentrations relative to all other dates for both water and sediment samples (Fig. 4). Concentrations decreased over time with lowest values recorded in December (approximately 10–fold decline). Multiple regression analysis revealed that chla was correlated with total N concentration (adj $R^2 = 0.86; p<0.05$) and discharge (adj $R^2 = 0.71; p<0.05$).

Bacterial abundance differed among dates and the pattern of differences was similar in water (Fig. 5A) and benthic (Fig. 5B) samples. Bacterial counts were highest in June. In August, bacterial abundance was lower than in June but significantly higher (p<0.05) than the other dates. Bacterial abundance was lowest when flow returned in November and December. Bacterial abundance in both water and sediment samples was largely driven by changes in chla concentration, temperature and discharge as determined by multiple regression analysis (Table 2). While an average of 97% of the variation in bacterial counts was explained by chla concentration alone, temperature and discharge also accounted for a significant amount of variation (>86%).

Enzyme activity

Microbial extracellular enzyme activity significantly differed among dates (Fig. 6). Four out of five enzymes demonstrated similar results; AG, BG, NAG and LAP had highest activity in June and least activity in September when stream discharge was zero (Fig. 6 A-D). Activity increased after flow returned in November but remained low
compared to June. Unlike the other four enzymes, highest LAC activity was recorded in September (Fig. 6 E). No significant differences in LAC activity were observed between the other dates (April, June, November and December) and were, on average, 4.6 fold lower than in September.

Multiple regression analysis indicates that temporal variations in environmental factors were strongly correlated with extracellular enzyme activity (Table 3). Changes in nitrate concentration over time had a profound effect on AG, BG, NAG and LAP activity. Nitrate concentration alone explained most of the variation in AG and NAG activity. Nitrate concentration, temperature, chla and humic concentrations were correlated with BG activity. Alterations in discharge and nitrate concentration were correlated with changes in LAP activity and seasonal changes in DO, conductivity and discharge accounted for variation in LAC activity.

**T-RFLP**

Redundancy analysis (RDA) revealed significant differences in bacterial community structure among dates while the RDA ordination plot with vectors illustrate the degree of correlation between environmental variables and bacterial community structure (Fig. 7). In June and August, communities varied along RDA axis 1 whereas; on the other dates (April, September, November and December) communities were spatially separated along RDA axis 2. April and September had distinct bacterial communities with strong correlation with stream discharge (Fig 7). Bacterial community structure in June and August were most similar as they clustered together and the observed
community structure was largely explained by temporal variability in DOM composition, chla and total N concentration. November and December had somewhat similar community structure with varying degrees of overlap. Turbidity and conductivity were responsible most of the variation in bacterial community structure in fall (November and December).

**Discussion**

Bacteria play an important role in the function of ecosystems (del Giorgio and Cole 1998) and DOM quality and composition impacts bacterial community structure and function (Wehr et al. 1999; Strauss and Lamberti 2002; Anesio et al. 2004). Bacterial communities are influenced by alteration in water availability (Romaní et al., 2006; Artigas et al., 2009) such as the seasonal pattern of hydrology in the study stream. Biotic interactions between bacteria and algae are important (Romaní et al., 2006; Ylla et al., 2009; Pohlon et al., 2010) and varied seasonally in this study. Both bacterial community structure and function varied seasonally as did environmental conditions (including DOM and stream hydrology).

Over time, the conditions of the stream varied in ways, which potentially impacted attributes of the bacterial community. Overall, nitrate concentration in LWD spiked in summer (June) and dominated the total N pool. Chl a concentrations in LWD had a strong correlation with nitrate concentration and is in agreement with the fact that increased N concentrations influence high algal production in streams (Horner 1983; Dodds et al. 1997). Decrease in chl a concentrations over time, in LWD, can be attributed
not only to decline in nitrate concentration but also to discharge because stream flow contributes to algal growth rates by stimulating nutrient uptake and metabolism (Stevenson 1996).

Enzyme activity in sediments is often correlated with organic matter content (e.g. Romaní and Marxsen, 2002; Rulík and Spáčil, 2004; Sinsabaugh et al., 2008; Artigas et al., 2009). This is corroborated by enhanced activities of cellulolytic enzymes like AG and BG, in LWD sediments with high concentrations of benthic chl a in June. Extracellular enzymatic activity in the present study was highly correlated with temporal variations in DOM concentration as well as stream discharge. Likewise, Gerull et al. (2011) found a correlation between chl a and BG. Perhaps, heterotrophs excrete enzymes to scavenge C of algal origin and the DOC from algae might serve as priming agent to facilitate enzymatic degradation of other recalcitrant carbon compounds (Guenet et al. 2010; Rier et al. 2007).

Variation in activity of the two N-acquiring enzymes, LAP and NAG, over time was very similar to that of C-acquiring enzymes, but unlike BG, LAP and NAG activity had a strong correlation to the nitrate concentration and discharge. Alteration in bioavailability of certain nutrients like N due changes in hydrologic and moisture regimes (Pinay et al. 2007; Sahrawat et al. 1980, Poff and Ward 1989) might have driven changes in these enzyme activities.

Temporal patterns in LAC activity (for degrading aromatic rings) were markedly different from the other 4 enzymes. With the decrease in algal growth in autumn, there is a decline in supply of labile organic matter as it gets consumed by heterotrophic
microbes in stream. This leaves behind recalcitrant organic matter pool composed of aromatic compounds. Enhanced LAC activity in September is reflective of degradation of such refractory compounds.

Fractionation of the DOM pool in this study revealed variation with time; the labile fraction increased in June which can be linked to high chl *a* concentrations, as autochthonous sources (such as algal exudates) are major contributors of labile DOM (Wolter 1982; Moran 1990; Sell and Overbeck 1992). With decline in algal growth, the labile fraction decreased leaving the refractory (humic) fraction that dominated the DOM pool from September to December. Bacterial abundance was largely driven by seasonal changes in chl *a* concentrations; bacterial counts were highest in June when chl *a* concentration was at its peak. DOM from autochthonous sources (like algal exudates) are considered labile (Wolter 1982; Moran 1990; Sell and Overbeck 1992) and can be readily assimilated by bacteria.

Examination of bacterial community structure revealed significant differences community structure driven by changes in biotic and abiotic factors over time. Distinct community structure in April and September that was largely driven by discharge, as changes in hydrologic and moisture regimes can alter in bioavailability of nutrients (Pinay et al. 2007; Sahrawat et al. 1980, Poff and Ward 1989). Bacterial communities were highly similar in June and August, which is attributable to similar DOM fractions (high proportion of labile fraction), that in turn was driven by chl *a* concentrations.

While enzyme activities of the C and N acquiring enzymes were related to changes in environmental factors, they were unrelated to the structure of bacterial
communities in LWD. However our result that community structure was unrelated to function is in line with other field studies that did not find evidence for a strong relationship between community structure and rates of ecosystem processes (Langenheder et al., 2005, 2006; Wertz et al., 2006; Östman et al., 2010). Lack of relationship between bacterial community structure and function (enzyme activity) can be attributed to functional redundancy of bacterial communities. Shifts in community structure would induce weak effects on functional properties (Östman et al., 2010) provided the dominant bacterial species are generalists in terms of the resource utilization in a given ecosystem. In such cases, depletion of a particular resource would enable generalist communities to benefit from a wide range of alternative resources (Egli, 1995) by switching metabolic pathways. Therefore functional redundancy is the most likely explanation for absence of congruent patterns among bacterial community structure and function (Burke et al. 2011).

In conclusion, the results from this study reveal that DOM composition was strongly related to algal growth which in turn was influenced by seasonal variation in N availability. Changes in both bacterial community structure and enzyme activity were largely driven by variation in environmental conditions (such as DOM and stream discharge). However, the lack of coupling between community structure and function was possibly driven by functional redundancy of the microbial community.
References


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allochthonous carbon sources of bacteria: results from whole-lake $^{13}$C addition experiments. Limnology and Oceanography 49: 588–596.


Romaní AM, Vázquez E, Butturini A. 2006. Microbial availability and size fractionation of


Physicochemical variables measured at Leary Weber Ditch, IN., on 6 different dates in 2011 (April, June, August, September, November and December). Values are means and standard errors.

<table>
<thead>
<tr>
<th>Date</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>Conductivity (µS/cm)</th>
<th>DO (mg/L)</th>
<th>Turbidity (NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>11.08±0.07</td>
<td>7.6±0.03</td>
<td>637.38±0.68</td>
<td>10.78±0.01</td>
<td>4.89±0.03</td>
</tr>
<tr>
<td>June</td>
<td>11.30±0.09</td>
<td>7.6±0.13</td>
<td>729.60±7.02</td>
<td>10.74±0.06</td>
<td>7.04±1.08</td>
</tr>
<tr>
<td>August</td>
<td>22.50±0.24</td>
<td>7.0±0.18</td>
<td>654.11±7.33</td>
<td>9.06±0.18</td>
<td>8.74±0.46</td>
</tr>
<tr>
<td>September</td>
<td>17.88±0.21</td>
<td>7.7±0.17</td>
<td>543.00±3.49</td>
<td>8.75±0.25</td>
<td>8.20±0.22</td>
</tr>
<tr>
<td>November</td>
<td>14.06±0.07</td>
<td>7.4±0.01</td>
<td>756.60±6.87</td>
<td>10.45±0.12</td>
<td>10.91±0.16</td>
</tr>
<tr>
<td>December</td>
<td>10.80±0.14</td>
<td>7.4±0.03</td>
<td>763.60±7.93</td>
<td>10.54±0.16</td>
<td>9.30±0.20</td>
</tr>
</tbody>
</table>
Table 2. Results of stepwise multiple linear regression that includes $R^2$, coefficients, and p-values for each significant variable that explains differences in bacterial abundance (as the dependent variable) over time.

<table>
<thead>
<tr>
<th>Bacterial abundance</th>
<th>Steps</th>
<th>Independent variable</th>
<th>$R^2$</th>
<th>$\Delta R^2$</th>
<th>Coefficient</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1</td>
<td>Chla</td>
<td>0.97</td>
<td>0.97</td>
<td>12.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Temperature</td>
<td>0.95</td>
<td>0.02</td>
<td>5.32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Turbidity</td>
<td>0.93</td>
<td>0.02</td>
<td>3.21</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Discharge</td>
<td>0.90</td>
<td>0.03</td>
<td>1.01</td>
<td>0.002</td>
</tr>
<tr>
<td>Sediment</td>
<td>1</td>
<td>Chla</td>
<td>0.97</td>
<td>0.97</td>
<td>22.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Temperature</td>
<td>0.96</td>
<td>0.01</td>
<td>6.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Discharge</td>
<td>0.87</td>
<td>0.10</td>
<td>2.2</td>
<td>0.03</td>
</tr>
</tbody>
</table>
**Table 3.** Results of multiple linear regressions (stepwise forward and backward selection) with microbial extracellular enzyme activity (nmol h$^{-1}$g$^{-1}$) as the dependent variable.

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Model factors</th>
<th>Adjusted $R^2$</th>
<th>p-Value</th>
<th>Coefficient</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-glucosidase (AG)</td>
<td>Nitrate</td>
<td>0.87</td>
<td>&lt;0.001</td>
<td>1.27</td>
<td>55.67</td>
</tr>
<tr>
<td>β-glucosidase (BG)</td>
<td>Chlα+ Humic + Temperature + Nitrate</td>
<td>0.98</td>
<td>&lt;0.05</td>
<td>93.24</td>
<td>166.29</td>
</tr>
<tr>
<td>N-acetylglucosaminidase (NAG)</td>
<td>Nitrate</td>
<td>0.75</td>
<td>&lt;0.001</td>
<td>33.85</td>
<td>188.11</td>
</tr>
<tr>
<td>Leucineaminopeptidase (LAP)</td>
<td>Nitrate + Discharge</td>
<td>0.77</td>
<td>&lt;0.001</td>
<td>1.03</td>
<td>57.04</td>
</tr>
<tr>
<td>Laccase (LAC)</td>
<td>DO + Conductivity + Discharge</td>
<td>0.94</td>
<td>&lt;0.05</td>
<td>48.03</td>
<td>64.28</td>
</tr>
</tbody>
</table>
Figure 1: Leary Weber Ditch discharge rate on six different dates. Letters on the bars indicate significant differences (p<0.05) between dates based on Tukey’s HSD post hoc multiple comparisons. Values are means and standard errors.
Figure 2: Total nitrogen (N) and nitrate concentration of Leary Weber Ditch water samples obtained on six different dates. Bars with different letters are significantly different from each other (p<0.05) between dates based on Tukey’s HSD post hoc multiple comparisons. Values are means and standard errors.
Figure 3: Dissolved organic carbon (DOC) concentration of fractions obtained by ion exchange chromatography of Leary Weber Ditch water samples collected on six different dates. The three fractions are as follows: Humic, Labile (cationic) and Labile (anionic).
Figure 4: Chlorophyll $\alpha$ concentrations in A) water and B) benthic, on six different dates. Bars with different letters are significantly different from each other ($p<0.05$) between dates based on Tukey’s HSD post hoc multiple comparisons. All values are means and standard errors.
Figure 5: Bacterial abundance in A) water and B) sediment samples (based on DAPI staining) collected on six different dates. Bars with different letters are significantly different from each other (p<0.05) between dates based on Tukey’s HSD post hoc multiple comparisons. All values are means and standard errors.
Figure 6: Microbial enzyme activity in Leary Weber Ditch sediment samples, expressed as nmol substrate converted per hour per g (dry mass) of sediment sample, collected on six different dates. A) α-glucosidase (AG) activity; B) β-glucosidase (BG) activity; C) N-acetylglucosaminidase (NAG) activity; D) Leucineaminopeptidase (LAP) activity and E) Laccase (LAC) activity. Bars with different letters are significantly different from each other (p<0.05) between dates based on Tukey’s HSD post hoc multiple comparisons. Values are means and standard errors.
Figure 7: Two-dimensional RDA ordination plot showing the relationship of bacterial community structure (T-RFLP profiles) with environmental parameters on different dates. Each vector points to the direction of increase for a given variable and its length indicates the strength of the correlation between the variable and the ordination scores for the community data.
CHAPTER 5

SYNOPSIS

It is important to understand the fate of nutrients, like N, in streams given that anthropogenic activity, such as agriculture, have increased inputs of biologically reactive nitrogen to the environment (Kemp and Dodds 2001; Royer et al. 2004) that deteriorates stream health and cause eutrophication. As mentioned in Chapter 1, microbes play a crucial role in the driving essential biogeochemical transformations; they possess the ability to convert a wide range of nitrogen compounds into forms that vary in their availability to other organisms (Madigan et al. 2006). The purpose of this research was to improve our understanding of bacterial organic and inorganic nitrogen utilization.

Resources (such as C and N) can change due to seasonal variation in physicochemical parameters, as well as biotic factors like habitat modification by organisms (Jones et al. 1994; Odling et al. 2003). One of the survival strategies of organisms in its natural habitat involves modification of the quantity and possibly the quality of available resources (Pekkonen and Laakso 2012). This two-way relationship between organisms and environment adds complexity to species interactions. Although, extensive research on resource-consumer dynamics and species interactions has been carried out till date, yet, the effects of alteration in resource environment on bacterial community are largely unknown (Pekkonen and Laakso 2012).

Resource partitioning is essential for survival of bacteria in natural communities. The amount of variability in microbial resource utilization can be dictated by either
narrow niche widths, with minimum variation in resources pool, supporting specialism by
broad niche widths, large variation in resource pool, supporting generalists (Roughgarden
1972). In chapter 2 we determined the pattern of organic and inorganic N utilization by
265 bacterial species in order to improve our understanding of factors that affect
generalist and specialist trait among bacteria. The differences in N utilization add in to
the evidence that bacteria coexist in nature by means of resource-partition (Bonsall and
Wright 2012). The isolates obtained from 8 different N enrichments and a strong
correlation between the initial enrichment they were derived from and their N utilization
pattern. Results also suggest that, an isolate can act as both generalist and specialist
depending on the enrichment it is isolated from. This finding is indicative of
microenvironment driven changes rather than taxonomic identity of bacteria.

However, bacteria do not exist as an individual organism in nature but is a part of
a rather complex community. Changes in natural community structure are mostly driven
by resources, broadly classified as: essential, complementary, switching, or antagonistic
(Tilman 1982). In Chapter 3, the effect of changes in resource source and availability
(more specifically N and C) on bacterial community was examined. Several layers of
complexity imposed by temporal and spatial variation in biotic and abiotic factors makes
it difficult to measure species interactions in natural (Laska and Wootton 1998) settings.
However, laboratory microcosms are controlled simplified resource environments with
nutrient amendments and have served as useful systems for understanding resource-
consumer dynamics (Lendenmann et al. 1996; Velicer and Lenski 1999; Friesen et al.
2004; Barrett et al. 2005; Mclean et al. 2005). To elucidate the effect of organic carbon
availability on bacterial uptake of organic and inorganic nitrogen, experiments were carried out in controlled laboratory conditions where bacterial communities colonized on glass beads were subjected to various N and C amendments. Resource types have been known to influence competition (Leon and Tumpson 1975); that in turn can alter bacterial community response to nutrient utilization (Rozen and Lenski 2000; Finkel 2006). This study demonstrated a strong link between bacterial C and N dynamics. C availability dictated bacterial uptake of organic and inorganic nitrogen (Seitzinger, 1994; Jones et al., 1995; Currie, 1999). Also DON composition (labile vs refractory) affected bacterial utilization of organic N. Significant differences in bacterial community in different C and N amendments suggest that C, DIN, and DON treatments drove changes in bacterial community composition (Vrede 2005), that, in turn, affected rates of DON and DIN utilization under various C concentrations (Reed and Martiny 2007). Though, these systems lack the complexity of natural environments, they can rapidly change due to bacterial interactions and metabolic response. Resource partitioning, competition and the release of secondary metabolites often bring about unpredictable changes in the resource composition, which in turn can affect community composition (Rozen and Lenski 2000; Finkel 2006).

Although, it is challenging to quantify community response to changes in the natural resource pool due to increased levels complexity under natural settings, it is important to understand the factors, such as temporal variation and fluctuation in physicochemical parameters, which affect growth and function of microbial communities (Odling et al. 2003) in streams. In aquatic ecosystems bacteria are exposed to an array of
nutrient sources along with factors (like competition, cross-feeding, metabolic capability etc) that mediates inter- and intra-specific interactions within a community (Madigan 2005; McArthur 2006) and also supports the existence of polymorphic populations (Rosenzweig et al. 1994; Chesson and Huntly 1997; Treves et al. 1998; Rozen and Lenski 2000; Friesen et al. 2004). In chapter 4 changes were tracked in bacterial community structure and function. While alteration in the environmental conditions drove differences in bacterial community structure, resource availability dictated their functional (extracellular enzymatic activity) response. However the lack of any link between bacterial community structure and function presumably was due to functional redundancy of the bacterial communities (Burke et al. 2011).

N cycle in aquatic environment constitutes of an array of complex microbially mediated transformations and heterotrophic bacteria play a vital role in such transformations depending on their metabolic capability. In this research, N utilization by bacteria was studied across a range with increasing levels of complexity; starting from isolates, to controlled laboratory microcosms followed by natural communities in stream sediment.

Overall, experimental evidence as described in this dissertation demonstrates that a suite of environmental variables control bacterial organic and inorganic N utilization. Increase in organic C concentration, enhances the metabolic demand for N by heterotrophic bacteria. Results of this study reveal that within the physical and chemical limits set by the constraining variables, bacterial preference for organic and inorganic N was largely driven by their growth environment (enrichment) and DOC availability. The
research presented here is an attempt to improve our understanding of bacterial C and N dynamics in aquatic ecosystems.
References


