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BIOGEOCHEMICAL CYCLING OF NITROGEN THROUGH A LANDSCAPE RICH IN LEGACY SEDIMENTS

A Thesis in

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by

Julie N. Weitzman

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The thesis of Julie N. Weitzman was reviewed and approved* by the following:

Jason P. Kaye
Assistant Professor of Soil Biogeochemistry
Thesis Adviser

Michael N. Gooseff
Assistant Professor of Civil Engineering

Richard C. Stehouwer
Associate Professor of Environmental Soil Science

David M. Sylvia
Professor of Soil Microbiology
Head of the Department of Crop and Soil Sciences

*Signatures are on file in the Graduate School.

ABSTRACT

Sedimentation rates, anoxic conditions, and eutrophication have all increased in the Chesapeake Bay since the time of European settlement. Legacy sediments, deposited during the historic, post-settlement period marked by deforestation, land clearing, and plowing of uplands and valley slopes, act as a significant non-point source of nitrogen to the Bay. At Big Spring Run in Lancaster, Pennsylvania, these legacy sediments now overlay a buried hydric soil, which affects the contemporary transfer of nitrogen from uplands to streams. Recent research suggests that nitrogen transfer to streams is also affected by soil drying and rewetting. Climate change models predict that the variability and magnitude of precipitation events will increase over time. Such changes could lead to extended periods of droughts, followed by increased precipitation. These dry-rewet cycles can alter the structure and activity of soil microbial communities, impacting nutrient retention and release. This project was undertaken to increase the understanding of nitrogen processing and movement in legacy sediments along a landscape gradient before and after soil drying.

Short-term incubations were carried out on field-moist soil as well as on soil that was air-dried, then rewetted prior to incubation. Samples were from three landscape positions (non-legacy uplands, legacy zone, and stream bank) and three layers (top 20 cm, midlayer, and bottom layer) . Respiration rates, net ammonification rates, and net nitrification rates were determined in laboratory incubations. A community-level physiological profile and extracellular enzymes were assayed to determine microbial activity levels. Four key discoveries were found from this work: net nitrification was greatest in the surface soils away from the stream bank; drought induced nitrate pulses were unexpectedly absent; buried hydric soils appeared to have low

biological activity and low nitrification potential; and the low activity in the buried layer may have been due to a lack of labile carbon.

Given their prevalence in Pennsylvania there was a critical need to understand how nitrogen flows through legacy sediments to improve predictions and management of nitrogen transport from uplands to streams. Future measurements are needed to explore how different hydrological flowpaths impact microbial communities and contaminant pulses in, and between, soil layers, and how such interactions change after the removal of legacy sediments. My data suggest that flowpaths through the buried hydric layer will not have strong nitrogen immobilization, despite high C in that layer.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER 1

Introduction and Objectives

Eutrophication is the leading cause of impairment in surface waters in the United States (Carpenter et al., 1998). While eutrophication is often attributed to contemporary nutrient pollution, there is growing evidence that past practices also are important. After European settlement, upland soil erosion due to forest clearing and plowing increased sedimentation rates to the Chesapeake Bay watershed (Brush, 2008). Much of this sediment was captured behind mill dams. A major new development in eutrophication research recognizes that these "legacy sediments" have altered nutrient cycling at the land-streamwater interface (i.e. the riparian zone) (Walter and Merritts, 2008). Legacy sediments likely alter eutrophication processes in two ways. First, as abandoned dams breach, stream bank erosion of legacy sediments occurs and acts as a significant non-point source of suspended sediment (and nutrients entrained in the sediment) (Trimble, 1997; Walter and Merritts, 2008). Second, legacy sediments that have not yet eroded affect the contemporary transfer of nutrients from uplands to streams. **The objective of this project was to increase the understanding of nitrogen (N) processing and movement in legacy sediments along a landscape gradient before and after soil drying.**

Introduction. Legacy sediments were deposited during the historic, post-settlement period due to intense land clearing, deforestation, and the construction of numerous milldams, which occur in high concentrations in the mid-Atlantic region, and constitute substantial volumes of sediment stored in stream corridors. Understanding how uplands, legacy sediment accumulation zones, and wetlands act to filter nitrogen before entering stream waters is important in predicting downstream effects of legacy sediment. To investigate the processing of nitrogen, I conducted a combination of field and laboratory studies. The data obtained by this

project will act as a crucial component in establishing new Best Management Practices for stream restoration in the Mid-Atlantic Piedmont region. Such an interdisciplinary study at the landscape scale, involving the interrelationships among soil physical, chemical, and biological characteristics, can be used to identify key processes that contribute to basin wide nutrient and sediment loads. Enhancing our understanding of the dynamic properties in soils in regard to nitrogen cycling will be useful for future decision-making related to soil and environmental health.

This project focuses on how regionally prevalent legacy sediments alter the transfer of nitrate from soils to streams. Lancaster County is recognized as a hotspot for high sediment and nutrient yields to the Chesapeake Bay, and bank erosion of legacy sediments is a major source of these pollutants (Merritts and Walter, 2003). Extensive, collaborative work has taken place over the past ~10 years at a site known as Big Spring Run (BSR). Sediment and nutrients that enter the streamwaters near BSR have the potential to flow into the Susquehanna River, and eventually travel all the way to the Chesapeake Bay. Lowering N inputs from non-point sources could lead to a reduction of eutrophication in the Bay. An important aspect of this project involves evaluating which forms of N are dominating the system, and how different conditions can affect N availability. BSR served as an ideal study location for tackling the objectives set forth in undertaking this research.

As in many stream banks of the mid-Atlantic Piedmont region, the stream banks along BSR consist of four principle stratigraphic units, which from bottom to top include: (1) basal gravels; (2) pre-settlement hydric soils; (3) post-settlement alluvium and colluvium (the latter informally called “Legacy Sediments”); and (4) newly developing A horizon (Walter and Merritts, 2008). The basal gravels, which are roughly 30 cm thick, are composed of angular to

subangular quartz cobbles, which are interpreted to derive from Pleistocene periglacial lag deposits. These gravels are overlain by a 30-50 cm dark, organic matter rich, hydric soil, which apparently formed in a fluvial wetland environment over the last 10,000 years (Merritts et al., 2003; Walter and Merritts, 2008). Legacy sediments were deposited on top of the hydric layer during the historic, post-settlement period marked by deforestation, land clearing, and plowing of uplands and valley slopes. This period of accelerated soil erosion coincided with the construction of numerous milldams in the mid-Atlantic. Dams created reservoirs that flooded valley bottoms and acted as efficient sediment retention ponds. Behind the former milldam on BSR, a gradient of legacy sediment depth now exists with sediments thickest near the location of the dam, and tapering off upstream away from the dam. In the top 20 cm of the legacy sediment, an organic matter rich A horizon is developing. Beneath the A horizon, and continuing down the surface of the hydric layer is a ~80 cm thick layer of legacy sediment with lower (relative to the A horizon above and hydric below) organic matter content. Given their prevalence in PA, there is a critical need to understand how N flows through legacy sediments to improve predictions and management of N transport from uplands to streams. This project was undertaken to fill that need by assessing nitrate retention and microbial activity in BSR sediment.

Microbial activity drives the global N cycle, as microbes have a central role in almost all aspects of N availability. The impact of drying-rewetting cycles on soil microbial communities is of particular interest in light of the predicted effects of climate change on weather patterns. Soil drying and rewetting is considered a common physiological stress for microbial communities (Fierer et al., 2003). Furthermore, drying-rewetting cycles have recently been linked to region-wide pulses of high nitrate concentrations in tributaries of the Chesapeake Bay (Kaushal et al., 2010). Microbiological characterization of soils includes analyzing such

parameters as respiration, carbon and nitrogen mineralization, substrate utilization, and enzyme activities (Pesaro et al., 2004). Short-term effects of the rapid rewetting of a dry soil have included increases in C and N mineralization rates (Birch, 1958). These short-term spikes in C and N mineralization rates may be due to the release of labile substrates during microbial cell lysis (Fierer et al., 2003). The physiological stress brought on by the rewetting of a dry soil could cause a reduction in the total soil microbial diversity or an increase. If a portion of the microbial community is favored because of its ability to adapt and cope with the stress, then microbial diversity could decrease (Fierer et al., 2003). If, however, the spatial and temporal heterogeneity of the soil environment is enhanced during the drying-rewetting cycles, then microbial diversity could increase within the soil (Fierer et al., 2003). Determining how soil microbial communities respond to such drying-rewetting events across depths and landscape positions is thus important for understanding how a shift in microbial composition could affect the release or retention of nutrients to nearby streamwaters.

Objectives. Biogeochemical N cycling in the stream bank sediments, riparian zones, and upland soils of the Big Spring Run (BSR) Watershed, Lancaster County, Pennsylvania was assessed in order to better understand the effect of N cycling and microbial activity on the filtration of nutrients. The overarching questions and hypotheses that drove this project included: (**1) Is there a difference in net nitrification rates in legacy sediment soils compared to upland soils?** I hypothesized that potential net nitrification rates will be greater in legacy sediment strewn soils than in upland soils. Legacy sediments are suspected to contain high concentrations of ammonium (Dr. Robert Walter, pers. comm.), the key substrate for nitrification, suggesting that soils composed of such materials will generate more NO_3^- than soils lacking legacy sediments. **(2) Where is nitrification naturally high in the soil profile of BSR?**

Naturally high rates of nitrification are hypothesized to be found in the surface layers at the site. From observation they were presumed to have the highest organic matter content (which can serve as a source of ammonium via mineralization), and due to their position, high oxygen levels. I hypothesized that the midlayer soils would have lower nitrification than the surface soils. The midlayer soils were perceived to contain less organic matter and lower nutrient levels than the surface layers above. Nitrification rates are hypothesized to be the lowest in the buried organic matter-rich hydric soils because of depleted oxygen at depth. In oxygen limited conditions denitrification would be the dominant process, not nitrification. **(3) How does the distribution of nitrification rates across landscape position (non-legacy upland, legacy zone, and stream bank) and depth (surface legacy sediment enriched in organic matter, subsurface legacy sediment low in organic matter, and buried hydric soil) respond to wetting and drying events?** Based on previous studies I hypothesized that nitrification will increase in the soil at all three landscape positions when rewetted after a drought period. This "nitrate pulse" seen after rewetting is also hypothesized to occur in all three soil layers at BSR. **(4) Is the long-buried hydric soil still biogeochemically active, and if so, does it act as a filter to remove N? Specifically, are responses to different carbon substrates and enzyme activities in the hydric soil fundamentally different than those in the upper two soil layers?** I hypothesized that hydric soils will be active in immobilizing N before entering the stream. I expect microbial response to C substrate additions and enzyme activity levels will be greatest in the hydric soil, confirming that the long-buried soil is still biogeochemically active. A number of analyzes were carried out using soil samples collected at BSR to test the above hypotheses. These experiments provided useful foundational data about N cycling in hydric soils overlain by legacy sediments.

CHAPTER 2

Background

To fully understand how nutrients are cycled at Big Spring Run a history of the site, including past land uses and human impacts, must be identified. A once widely used practice in the Lancaster County area involved damming streams. Such modifications to the landscape impacted sedimentation rates, and in the process, natural internal nutrient cycling in the region. N cycling is of greatest interest at BSR because of the potential impacts the leaching and runoff of different N forms may have on downstream waters, including the eutrophication of the Chesapeake Bay.

Human Impacts on the Land and the Damming of Streams

Dams, Ponds, and Reservoir Sediments. The appearance of Big Spring Run today is drastically different than its appearance prior to European settlement. Before colonization, Big Spring Run was likely a series of small anabranching channels that flowed through islands of vegetated wetlands (Walter and Merritts, 2008). The pre-settlement wetland of Big Spring Run, which developed within the last 10,000 years, stored large amounts of organic rich material, but little sediment due to the low, long-term erosion rates in pre-settlement times (Walter and Merritts, 2008b). The valley bottoms of the watershed once consisted of a stream network with thin gravel layers over the carbonate bedrock, with soil horizons of organic-rich silty clay, which supported marshy vegetation (Merritts et al., 2005).

Anthropogenic alterations of the landscape have impacted both the geomorphology and hydrology of the land. It is believed that of all the human modifications to the land, two distinct practices have caused the greatest impact: the acceleration of upland soil erosion through agriculture, land-clearing, and other building efforts, and the construction of impoundments

along waterways, creating new sediment sinks (Renwick et al., 2005). Combined, these two modifications have transformed fluvial transport of sediments by increasing both the rate of sediment input and the number of sediment sinks. Such changes have significantly impacted the hydrological and ecological systems of both upstream and downstream environments (Doyle et al., 2005, Meade et al., 1990).

Dams serve many functions, being useful for such diverse purposes as irrigation, transportation, and flood protection. Above all, however, the construction of dams offers renewable energy sources (Cantelli et al., 2004). Dam building for water power in the United States began after the European settlement, beginning in the late 17th century and persisting through the early 20th century, declining with the adoption of the steam engine (Walter and Merritts, 2008). The most common types of dams were built for milling, and most spanned entire valley bottoms of dominantly 1st to 3rd order streams, rising to heights that averaged about 2.5 m high (Walter and Merritts, 2008). The most significant impacts of these dams involved trapping of sediments, which affects watersheds upstream and downstream of the impoundments (Meade et al., 1990). Although sedimentation behind large dams is more conspicuous, small impoundments, like the milldams of the eastern United States are more numerous, and thus have the potential for larger cumulative impacts (Renwick et al., 2005).

The National Inventory of Dams (NID) includes over 75,000 large dams. This number, however, only accounts for 1-2% of the existing impoundments in the United States (Renwick et al., 2005). Millions of smaller impoundments that have been constructed relatively recently, in the past few decades, are excluded from the NID, and the inventory does not include numerous milldams from early American history. Renwick et al. (2005) estimate that the construction of these modern, smaller dams, in addition to the larger dams still in operation, increased the total

sediment storage to about 14 x 10^9 m^3 per year between 1950 and 2000. Dams have fragmented every large river, disconnecting the once free-flowing, integrated water system (Graf, 1999). Large impoundments alone cannot account for this, and thus it is important to understand the potential that small dams have for sediment storage.

Sediment Sources, Sinks, and Transport. In general, most upland sediment derived from accelerated erosion is still present on the landscape in the stream corridors (Meade, 1982). Instead of being transported out of the system and deposited in environments much farther downstream from erosional sites, a large fraction of the sediments are being deposited and stored along the stream banks. It is estimated that 66-94% of all fluvial sediment remains in storage, while the relatively small remainder is exported through the system (Costa, 1975; Trimble, 1983; Phillips, 1991; Beach, 1994). Thus, as vast majorities of sediment are still in storage in stream corridors and subject to erosional processes that can cause the gradual, long-term remobilization of this sediment, a constant throughput of sediment can be provided to stream systems (Renwick, 2005). Storm events, or the removal of dams, can cause substantial erosion of impounded sediment in alluvial and colluvial settings (Doyle et al., 2005). Such remobilization can affect water quality through the downstream transport of sediment-bound contaminants, or through the alteration of biogeochemical cycles (Hart et al., 2002). While little is known about the downstream fate of transported sediment, the ecological effects of high suspended sediment loads are known and far reaching (Doyle et al., 2005).

Consequences of Dam Removal. Dams and their impoundments can trap >50% of sediment that enters their reservoirs, delaying the movement of these materials through the water system (Graf, 1999). Dam removal and flushing of stored sediment can have extensive consequences, influencing ecosystems and hydrological systems at large spatial and temporal

scales (Hart et al., 2002). Dams tend to be removed when they outlive their usefulness. When the reservoirs behind hydroelectric dams fill with sediment for example, the dam's ability to store water and produce electricity diminishes, which can lead to its removal (Cantelli et al., 2004). Dam removal releases stored sediments, which can degrade downstream habitats (Cantelli et al., 2004). After dam removal or breaching, most stream channels evolve in a similar way (Pizzuto, 2002). First, the channel will incise into the previously impounded sediment column. Following this initial incision, the stream propagates laterally, and depending on the strength of the channel's stream banks, will soon lead to bank erosion and channel widening. This supply of coarser sediments from bank erosion causes aggradation of the streambed by mostly gravels and sands, while silts and clays are transported in suspension through the stream system (Pizzuto, 2002). The evolution of streams after dam removal can occur over several decades (Pizzuto, 2002). Initially a large pulse of remobilized sediment will be delivered to the stream, and over time, the incising processes of the stream will cause sporadic fluxes of suspended sediment from bank collapse. This long-term sedimentation of the channel will continue to affect the hydrological and ecological systems downstream (Doyle et al., 2005).

Big Spring Run Study Site

Geologic History of Legacy Sediment Deposition. As in many stream banks of the mid-Atlantic Piedmont region, the stream banks along Big Spring Run consist of four principle stratigraphic units, which from bottom to top include: (1) basal gravels; (2) pre-settlement hydric soils; (3) post-settlement alluvium and colluvium (the latter informally called "Legacy Sediments"); and (4) newly developing A horizon (Walter and Merritts, 2008). Each unit formed under different conditions and over different periods of time. At Big Spring Run, the basal gravels, which are roughly 30 cm thick, are composed of angular to subangular quartz cobbles,

which are interpreted to derive from Pleistocene periglacial lag deposits. These gravels are overlain by a 30-50 cm dark, hydric soil, which apparently formed in a fluvial wetland environment over the last 10,000 years (Merritts, et al., 2005; Walter and Merritts, 2008).

Legacy sediments at Big Spring Run were deposited during the historic, post-settlement period marked by deforestation, land clearing, and plowing of uplands and valley slopes. This period of accelerated soil erosion coincided with the construction of numerous milldams along Big Spring Run. These dams created long, linear millpond reservoirs that flooded valley bottoms several kilometers upstream, which became efficient sediment retention ponds. Behind one former milldam at the mouth of Big Spring Run, a gradient of legacy sediment depth now exists with sediments thickest near the location of the dam, and tapering off upstream away from the dam. Vegetative roots, extending about 5 cm down at the Big Spring Run site, were found in the developing A horizon. Dominated by mineral particles, the A horizon has a darkened appearance due to the accumulation of organic matter. Beneath the A horizon and continuing down the stratigraphic column a pale, yellowish-brown sequence of clay, silt, and fine sand can be observed. Underlying this light brown layer is a dark brown layer containing orange, oxidized iron stains. The deepest soil is a dark, organic-rich silt loam layer and interpreted as being a buried hydric (wetland) soil (Walter and Merritts, 2008). The underlying basal gravel layer, consisting predominately of quartz, rests on weathered bedrock of the Conestoga limestone, which forms a planar valley bottom.

Stream bank erosion and upland nutrient runoff serve as the main pathways for nutrients to enter (and reenter) waterways. Leaching is also a principal pathway through which nutrients can be lost from an environment. Losses of N by leaching can occur when soils have more incoming water than the soil can hold. NO_3^- present in the soil solution will be carried along

with the excess water, while NH_4^+, which has a positive charge, will be held by negative sites in the soil. Thus, NO_3^- leaching into the groundwater or nearby stream systems is of more concern than possible NH_4^+ leaching. This study seeks to better understand the possible role that the different soil layers at Big Spring Run have for filtering N, and how different flow paths can alter such filtration abilities.

An increased understanding of the composition and fate of N in surface soils, riparian zone legacy sediments, and pre-settlement hydric soils will provide a better understanding of significant pools of nitrogen, and how its inputs into streams can affect the ecology of downstream aquatic systems.

Study site: ***Geologic and Geographic Setting.*** This project utilized soils collected along the Big Spring Run Watershed of Lancaster County, Pennsylvania. This site is an optimal location because it is an already established study site for many scientists, and has a wide range of available data. BSR acts as an infrastructure for research and education because a growing group of researchers, students, and stakeholders are focusing efforts on this watershed as a primary case study in science-based legacy sediment restoration. This project benefited from the development of partnerships with the many stakeholders already involved in the monitoring of BSR, including F&M College, USGS, USEPA, DEP, PA Division of Dam Safety, the Chesapeake Bay Commission, and LandStudies, Inc.

Big Spring Run is a northward-flowing tributary of Mill Creek situated in West Lampeter Township, in central Lancaster County, Pennsylvania. Big Spring Run is primarily used for livestock watering, aquatic life support, and fish and wildlife support (Lathrop et al., 2007). The uncontrolled access of livestock to Big Spring Run and its stream banks, as well as the application of nutrients to adjacent pastures and upland farms, are all thought to contribute

significant amounts of non-point source pollutants. The Pennsylvania Department of Environmental Protection estimates that grazing animals along the watershed deposit an average of 8 pounds of phosphorus and 40 pounds of nitrogen per animal per year (Lathrop et al., 2007). Other sources of excess nutrients, such as commercial, urban, or septic sources, are considered insignificant compared with that contributed by agriculture.

In 2002 Big Spring Run was listed as an impaired stream on the 303d impaired waters list for high nutrient and sediment loads (U.S. EPA). In the headwaters of the stream, the channel is actively eroding, and approximately 1.4 m of stream bank sediments are exposed, the upper 0.9 m of which are legacy sediments. These stream bank sediments, including the legacy sediments that were deposited behind a 3 m high mill dam that once existed about 2 km downstream, are remobilizing via bank erosion. Bank erosion occurs by undercutting, calving, massive block slumping, and "peeling", each of which can be observed along the Mill Creek watershed (Merritts et al., 2007). Flow patterns along BSR also have the potential to increase N pollution along and within the nearby streams. Depending on how soil water interacts with legacy sediment or hydric soil, different scenarios of nutrient loading to the area can occur.

The majority of Big Spring Run is in a backwater environment that is upstream of a once existent millpond that formed as a result of damming. The length of Big Spring Run is characterized by low velocity stream flow and reveals an accumulation of water, like that of a slack-water environment. The uniform, fine-grain size of the post-settlement alluvium (dominantly silt-clay, with massive, occasionally horizontal bedding) along the stream banks lends further support to the claim that the sediment at Big Spring Run is being deposited in very low velocity water. Such conditions argue against the idea that these sediments are being deposited as a floodplain, as none of the characterizations of floodplain deposits were observed

(i.e. fining-upbed grain deposits, etc.). Furthermore, preliminary pollen analysis of pre- and post-settlement deposits shows vegetation consistent with through-flowing water conditions during pre-settlement times versus stagnant, slough-like conditions in post-settlement times (Merritts et al., 2005). These pollen data are consistent with the geomorphological and sedimentological interpretation that sediment at Big Spring Run was deposited in a backwater environment of a millpond, slack-water system.

Big Spring Run lies within the Piedmont physiographic province, as does all of Lancaster County. The geology of the Mill Creek watershed consists of deep, well-drained silt-loam soils underlain by carbonate bedrock (Lathrop et al., 2007). This carbonate bedrock underlying Big Spring Run is mostly a Paleozoic, weakly metamorphosed, silty limestone of the Conestoga Formation (Merritts et al., 2005). The dominant soil type in the watershed is Newark silt loam, a somewhat poorly drained soil, with moderate permeability, high water capacity, and tight compaction, consequently soil runoff would be minimal, and at a very slow pace (Custer, 1985). Long-term weathering of this Piedmont landscape has produced a thick soil that is rich in silt, clay, and mica flakes, with some traces of iron hydroxides and oxyhydroxides (Merritts et al., 2005). From previous analysis on a soil pit near the headwaters of Big Spring Run, the sediments had an average pH of 6.6 (Sullivan, 2006). This recorded pH value is within the expected range of the region (Custer, 1985).

Rationale and Significance. Understanding current N-cycling is necessary to predict future N-cycling under different conditions. The release of legacy sediments via stream bank erosion, in addition to upland erosion and nutrient runoff, serves as the pathway for suspended sediments and nutrients to enter (and reenter) waterways. Through the study of stream banks,

riparian zone legacy sediments, and upland soils, non-point sources of nitrogen can be better understood.

A portion of Big Spring Run has been proposed by the Pennsylvania Department of Environmental Protection as a test site for implementing and monitoring a new "floodplain and wetland restoration" Best Management Practice (BMP) (Hartranft, 2007). Fitting the definition of legacy sediment, while also being located in a rural setting experiencing little land-use change, Big Spring Run, already a site for which much baseline data has been obtained, was a natural choice as a BMP test site (Legacy Sediment Workgroup, 2006).

The research undertaken for this project will provide an important step in predicting how the long buried wetland soil may respond to restoration. It will also contribute valuable information to assess how nutrient loads would react in the face of the proposed BMP. Implementing the restoration and management strategy will target legacy sediment, while also quantifying benefits versus cost for future projects (Hartranft, 2007). Legacy sediments will be removed throughout a portion of the Big Spring Run watershed, which will expose the buried wetlands and reconnect the original floodplain hydrology of the site.

Nitrogen Cycling

Biogeochemical Cycling of Nitrogen. Models of the terrestrial N cycle often distinguish external and internal components (Kaye and Hart, 1997). The external is composed of those processes that add or remove N from ecosystems. These processes include dinitrogen fixation, dry and wet N deposition, N fertilization, N leaching, runoff erosion, denitrification, and ammonia volatilization. The processes that convert N from one chemical form to another or transfer N between different pools are those that make up the internal N cycle. Processes involved in the internal cycling of N include plant assimilation of N, the return of N to soil in

both plant litterfall and root turnover, N mineralization, microbial immobilization of N, and nitrification (Hart et al., 1994). These later three processes that internally cycle N are of special interest in this project.

Understanding which factors control the rates of internal N-cycling is important because these processes can affect ecosystem structure and function, as well as environmental quality (Hart et al., 1994). Transformations between organic and inorganic N form a central part of the internal soil N cycle (Norton, 2000). Mineralization is the microbial conversion of organic N to inorganic N as either NH_4^+ or NO_3^-. Ammonification describes the conversion of organic N to NH_4^+, while nitrification is the conversion of NH_4^+ to NO_2^- and then NO_3^-. Immobilization is the assimilation of inorganic N by microorganisms.

Denitrification in riparian zones and wetlands. Denitrification is the reduction of nitrogen oxides, nitrate (NO_3^-), and nitrite (NO_2^-), to the gases nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2). This process is essential at all scales of life, from the ecosystem, to landscape, regional, and global scales. Denitrification is important to primary production, water quality, and the chemistry and physics of the atmosphere (Groffman et al., 2006). Noted as a key regulator of water and air quality, denitrification can lead to decreases in nitrogen additions to waterways by filtering forms of nitrogen that are highly reactive. Denitrifiers are heterotrophic facultative aerobic bacteria that reduce organic compounds in order to obtain energy. They are able to use oxygen in aerobic conditions and nitrate in anaerobic conditions as electron acceptors (Jansson et al., 1994). Thus, denitrification mainly takes place in reduced environments of sediments when the reduction of nitrate is carried out in the absence of oxygen, and organic matter is easily accessible. In addition to specific oxygen concentrations, denitrification can be limited by the supply of nitrate or carbon within the sediment, affecting nitrogen removal rates

(Hill, 1996).

Recent studies of subsurface soils along upland-riparian-stream continuums indicate that buried horizons in the riparian zones are carbon-rich and can act as "hot spots" of microbial activity (Gurwick et al., 2008a; Hill et al., 2004). It was originally hypothesized that buried horizons that were comparatively older and deeper would contain more decomposed organic matter, and thus would have less microbially available C than younger and shallower buried horizons (Gurwick et al., 2008a). Patterns of C mineralization found in the riparian subsurface, however, suggest that soil organic matter quality in buried horizons depends on the conditions during horizon formation, not on age or depth. Supplies of microbially available C strongly influence microbial activity, which in turn affects the ability of the buried horizons to support denitrification (Gurwick et al., 2008a).

In riparian zones denitrification acts as an important mechanism through which nitrate is removed from subsurface waters (Hill, 1996). Forming an important transition zone between land and freshwater systems, stream riparian zones encompass the strip of land between the stream channel and the hillslope (Hill, 1996). Because of their locations, these areas have a significant potential to regulate the movement of materials from uplands to streams, and are thus sometimes referred to as buffer zones (Castelle et al., 1994). Within the riparian zone, it is widely accepted that there are two interfaces (Triska et al., 1993). The first interface is at the upland perimeter. This is where materials enter the riparian zone and are transported towards the stream in surface runoff and groundwater. The second interface, also known as the hyporheic zone, is adjacent to the stream channel. Within this area is the subsurface zone in which stream water and groundwater mix (Hill, 1996). Riparian zones consist of very complex environments. Hydrology, sediment characteristics, and biogeochemical processes can be very different due to

the spatial heterogeneity of both the horizontal and vertical dimension within a single riparian zone (Hill, 1996). Due to this wide variability, it is important to set up a grid system for sampling in order to understand how hydrology and the water chemistry may interact with the riparian zone soil to influence denitrification rates.

Similar to riparian zones, wetlands are transitional lands between terrestrial and aquatic systems. The water table however, is usually at or near the surface in wetlands, or covered by shallow water (Cowardin et al., 1979). To be classified as a wetland, it must have one or more of the following three attributes of the substrate: 1) hydrophytes as the mainly supported vegetation type; 2) consists predominately of undrained hydric soil; and 3) considered non-soil and is saturated with or covered by shallow water. Essentially, wetlands are lands where saturation with water is the dominant factor that determines the nature of soil development as well as the types of plant and animal communities that exist and thrive in the area (Cowardin et al., 1979). Denitrification is known to be an important process in wetlands for mediating inorganic nitrogen inputs from the landscape (Elliott and Brush, 2006). Restoration and establishment of wetlands have been suggested as cost-effective measures to reduce legacy sediment nutrient pollution because they are known to retain nitrogen and limit its transport in streams (Jansson et al., 1994; Carpenter et al., 1998).

Role of Microbes and Drought. A burst of respiration and release of nitrogen has been long known to occur after a dry soil has been rewet (Xiang et al., 2008). This release of mineralizable C and N on rewetting is known as the "Birch effect" (Birch, 1958). The repeated drying-rewetting cycles that occur in the soil impact important soil processes such as aggregation, soil organic matter decomposition, and nutrient cycling (Mikha et al., 2005). A characteristic pattern of decomposition has been noted to occur in both laboratory and field

conditions when a dry soil is moistened. The magnitude of decomposition, however, is dependent upon the percentage of carbon in the soil, and the climate (hot or cold) under which the soil is dried (Birch 1958).

The global N cycle is driven by the activities of microbial communities, which have been shown to be strongly influenced by a number of factors, including soil temperature, moisture, pH, or a combination of the four (Clark et al., 2009). Microbial biomass and organic residue can affect mineralization rates during dry-wet cycles (Mikha et al., 2005). During drought periods microorganisms may invest substrates into acclimation, become inactive, or even die due to the difficult living conditions (Muhr et al., 2010). As drought occurs, microbial C and N mineralization will decrease. Reduced water content also decreases substrate diffusion and thus substrate accessibility for microorganisms further decreasing mineralization rates (Muhr et al., 2010). Drying can also alter soil structure and the characteristics of soil surfaces. New substrates may become available due to aggregate disturbance and substrate desorption. Under drought stress microbial cells dehydrate and decrease the osmotic potential in the cell. To equilibrate with their environment, the microbes will then accumulate compatible solutes such as amino acids, carbohydrates, polyols, and inorganic solutes to maintain the osmotic potential in the cells (Borken and Matzner, 2009). Decreases in microbial biomass during drought, however, depend on the duration of drying, with larger reductions occurring over longer periods of drought.

Wetting of dry soils generally increases the microbial activity, causing a pulse of mineralization which can occur within minutes to hours (Muhr et al., 2010). The extent of such mineralization, however, can vary depending on soil properties, intensity and length of the drying, intensity of the rewetting, etc. (Borken and Matzner, 2009). As mentioned above,

microbial cells must accumulate compatible solutes in order to retain water and survive droughts. Upon rewetting the water potential increases rapidly, forcing cells to dispose of the solutes or risk cell rupture (Xiang et al., 2008). The compatible solutes are thus set free, and together with other readily degradable organic compounds from dead microbes the materials can be easily mineralized by any surviving microorganisms. This mechanism, referred to as the "microbial stress" mechanism, postulates that the C and N mineralization pulses seen after rewetting dry soils are due to the mineralization of substrates that were already present within the microorganisms. The substrates, however, were not previously available, as they were needed for purposes other than metabolization (Xiang et al., 2008; Muhr et al., 2010). A second mechanism, known as the "substrate supply" mechanism, is based on the idea that physical processes during rewetting of dry soil can destabilize soil organic matter (Xiang et al., 2008). Aggregate disruption, organic matter redistribution, etc. can create new substrates that were not available until the drying-rewetting event occurred, supplying new pulses of substrates to the surviving microbes.

Nitrate (NO_3^-) Flushing. Drought conditions followed by wet conditions coincide with pulsed watershed N exports (Kaushal et al., 2010). Contaminant loads in streams and rivers has increased as land is converted to human-dominated uses. Land use change has also transformed hydrologic cycles, which interact with contaminant pulses in unexpected ways (Vitousek et al., 1997). Land use change has also been associated with loss of floodplain wetlands which can lead to a decrease in the retention capacity of contaminants (like NO_3^-), an increase in erosion, and has the potential to contribute further to pulses of contaminants downstream (Kaushal et al., 2010).

Nitrate losses are of high concern because they have increased more consistently, and to a

greater extent, than any other ions found in disturbed systems (Vitousek et al., 1979). Nitrogen is frequently a limiting element for plant growth, and thus understanding its cycling is of critical importance. The release of hydrogen ions in the formation of NO_3^-, and the high mobility of the NO_3^- anion itself, promote the mobilization and loss of different soil cations that may increase pollution (Vitousek et al., 1979). High NO_3^- concentrations in runoff or groundwater can thus deleteriously affect downstream water quality.

Droughts can lead to accumulation of contaminants in watersheds, exacerbating the release of NO_3^- upon rewetting, which can enter nearby waterways. This phenomenon in which high concentrations of NO_3^- are released following a cycle of drying then rewetting is referred to as NO_3^- flushing. Release of NO_3^- can be affected by many different environmental conditions. Storage of N during dry years, mineralization of organic matter that has been washed into valleys, decreased NO_3^- removal via denitrification in groundwater or streams, or even increased biotic NO_3^- production in soils due to small drying and rewetting events can all change the magnitude and frequency of NO_3^- pulses (Kaushal et al., 2010).

The interaction of contaminants with legacy sediment is an area of research that is important for the future. Data regarding changes in contaminant pulses, especially that of NO_3^- flushing through legacy sediment can be used to guide river floodplain and wetland restoration strategies. Further data on contaminant pulses following drying and wetting events can also be used to improve forecasting in similar situations (Kaushal et al., 2010). Region wide NO_3^- flushing has been observed, but it is still uncertain how the NO_3^- in surface soils enters nearby streams (Kaushal et al., 2010). Increased study of contaminant pulse events, in particular those interacting with legacy sediments, is needed to protect ecosystem functions that have been affected by the changing magnitude and frequency of nutrient flushing.

Eutrophication of the Chesapeake Bay. Eutrophication, the overenrichment of nutrients in aquatic ecosystems, can lead to algal blooms and a loss of oxygen to bottom waters (Carpenter, 2005). Nitrogen, needed for protein synthesis, and phosphorus, needed for DNA, RNA, and energy transfer, are both key limiting nutrients required to support aquatic plant growth (Conley et al., 2009). It is widely accepted that estuaries and coastal marine ecosystems that have been heavily loaded with nutrients can display phosphorus limitation, nitrogen limitation, or co-limitation (Conley et al., 2009). Chemio-physical attributes, such as pH and the presence or absence of nitrogen-fixing bacteria, can cause one nutrient to become more critical than the other in limiting a system (Sterner and Hessen, 1994). While both N and phosphorus are strongly linked to eutrophication, studies of phosphorus have been abundant, resulting in decreased phosphorus loading to many waterways (Jansson et al., 1994). Even though phosphorus inputs to streams have been on the decline due to better point source management, nitrogen inputs have continued to increase. The increases in nitrogen loading have been linked to the precipitation of airborne nitrogen originating from combustion in traffic and industrial processes, the intensive use of nitrogen fertilizer, and changes in agricultural and silvicultural practices (Jannson et al., 1994). It is thus important to investigate nitrogen in stream banks and riparian zones loaded with legacy sediments. The potential these sediments have to release available forms of nitrogen is necessary to understand the role such sediments may play in exacerbating eutrophication downstream.

Studies of the eutrophication of the Chesapeake Bay – the nation's largest estuary – are more extensive than any other coastal ecosystem (Boesch et al., 2001). While excess phosphorus and nitrogen inputs from point sources, like sewage treatment plants, have been addressed and are declining due to the implementation of water-quality standards, non-point source pollution to

the Bay is still an important and intractable problem (Bennett et al., 2001). Non-point source pollution originates from diffuse, intermittent sources and is difficult to identify. However, the major source of nitrogen to freshwater systems, like the Chesapeake Bay is the non-point source flux of nitrogen from runoff, infiltration, and leaching into the water system (Sharpley et al., 1994). However, riparian vegetation has been shown to significantly reduce non-point nutrient flows to surface waters (Hill, 1996). Restoration of wetlands and floodplains are also thought to increase denitrification at the landscape scale, which can decrease the flow of nitrogen to downstream ecosystems, and thus reduce nitrogen pollution (Jansson et al., 1994).

Linking Nitrogen and Legacy Sediments. Soils along, and within, BSR are known to carry large concentrations of nutrients, such as nitrogen. It is important to measure the different types of nitrogen, not just the total amount of nitrogen, because its cycling is so complex. Nitrogen gas (N_2) in the atmosphere makes up the largest pool of nitrogen. In relation, the biologically-driven conversion of N_2 to chemically available forms of nitrogen, like NH_4^+ and NO_3^-, makes up a small pool. The pool of recycled nitrogen must also be accounted for, as it moves between the land, water, and atmosphere interface – cycling through the biosphere, pedosphere, hydrosphere, lithosphere, and atmosphere (Vitousek et al., 1997). Thus, an understanding of the composition and fate of nitrogen in eroded and stored legacy sediments and pre-settlement hydric soils will provide a better understanding of how nitrogen inputs into streams have changed and can affect the ecology of downstream aquatic systems. This project utilized a combination of field as well as laboratory studies, in order to analyze how nitrogen is retained and filtered within different soil sediments.

CHAPTER 3

MATERIALS AND METHODS

Study Site: This research was conducted at Big Spring Run (latitude: 39°59' N, longitude: 76°15' W), a northward-flowing tributary of Mill Creek, in Lancaster County, Pennsylvania. Big Spring Run lies within the Conestoga River Basin, which empties into the Susquehanna River, which provides about 50% of the freshwater entering the Chesapeake Bay (Chang, 2003; DEP, 2011). The site has a typical humid temperate climate, with precipitation a bit higher during summer months due to a higher frequency of convective storms. Soils along Big Spring Run consist of deep, silt-loam soils derived from limestone of the Conestoga Formation (Merritts et al., 2005). Big Spring Run is a small, almost entirely agricultural watershed. Due to high fertilizer and manure concentrations the area has been recognized as a significant non-point source of N to the Chesapeake Bay (Hall et al., 1997).

Basic Soil Characteristics: In April 2010, 29 soil cores (4.7 cm diameter, depth to refusal) were collected from Big Spring Run (Figure 1) in two differing landscape positions: (1) upland, non-legacy; and (2) legacy zone. Cores were divided into 20 cm increments and samples were taken at three depths: (1) surface; (2) midlayer; and (3) bottom. Surface samples consisted of the first 20 cm of the soil cores, and the samples from the midlayer and bottom soils were taken from the middle of each layer to reduce boundary effects. The average depth of midlayer soils was 40-60 cm, and 100-120 cm for bottom soils. Samples were weighed, homogenized by hand, and subsampled for the following analyses.

Potential C mineralization and potential net N mineralization were estimated using 7-day laboratory incubations. For the purposes of this study potential N mineralization was analyzed in terms of potential net nitrification and potential net ammonification rates (mg/kg soil/day). A

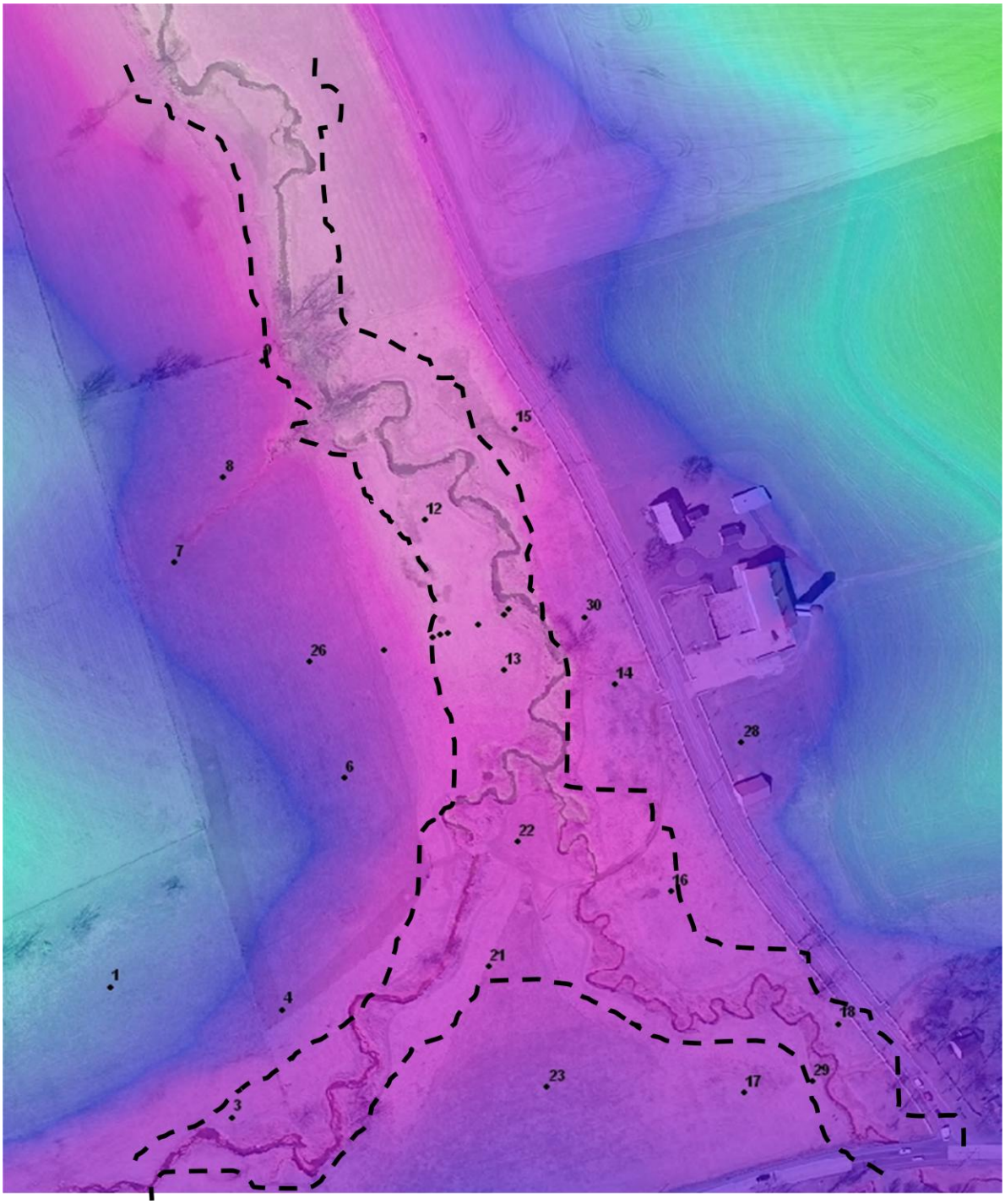

Figure **3-1:** GIS converted DEM map of Big Spring Run sampling sites taken in April 2010. Each dot and number grouping represents a core location. The dashed lines around the stream depict the estimated area over which legacy sediments were deposited. Any cores collected within the borders of the two dashed lines were classified as legacy zone soils. All cores taken outside the dashed lines, away from Big Spring Run, were classified as non-legacy upland soils. Stream bank samples were collected in September 2010 and made up the third classified landscape position – stream bank.

subsample of fresh soil was sieved (2 mm) and oven dried (105°C) to constant mass to determine the gravimetric water content. Another subsample of fresh soil was immediately extracted (100 mL of 2.0 *M* KCl). Two more subsamples of fresh soil were placed in 120 mL glass Wheaton vials. One of these vials was left unsealed to air-dry for 7 days. The other vial was immediately sealed with a septum. Empty Wheaton vials were also sealed in order to account for ambient CO_2. After 1-2 days a syringe was used to mix and sample the headspace gas generated in the vials. The concentration of CO_2 in a 1 mL headspace sample was determined using an infrared gas analyzer (LI-COR-7000). Before the vials were resealed they were fanned with ambient air in order to provide a uniform background CO_2 concentration. This CO_2 sampling protocol was repeated until the 7-day incubation period was at an end.

The samples in the Wheaton vials that were left to air-dry for 7 days were weighed each day to account for water loss. After the 7-day drying period, the "air-dry" vials were brought back up to their fresh weights using deionized water. Samples were fanned with ambient air, sealed, and their headspace sampled. The same sampling protocol as that used for the "fresh" samples was then carried out for the "air-dry, rewet" samples. The total incubation period for the "air-dry, rewet" was thus 14 days; 7 days of air-drying, then 7 days of laboratory incubation. Headspace concentrations for all samples were converted to μg C g soil^{-1} using the ideal gas law. Potential C mineralization for the "fresh" and "air-dry, rewet" samples was calculated as total C released over the incubation period.

Following the final headspace gas sampling in the "fresh" and "air-dry, rewet" soils, inorganic N was extracted for each sample. Ammonium and nitrate concentrations (as μg N g soil^{-1}) were determined on the initial and final "fresh" and "air-dry, rewet" KCl extracts using a spectrophotometer microplate reader. Rates (μg N g soil^{-1} day^{-1}) were determined by dividing

the concentrations by the total incubation period; 7 days for "fresh" and 14 days for "air-dry, rewet". Potential net N mineralization was calculated as the inorganic N concentration of the incubated subsamples minus the inorganic N concentrations of the initial extract. Separating the two inorganic N forms, potential net nitrification and potential net ammonification rates were also calculated. Pulses of C and N were determined by subtracting the "fresh" rates from the "air-dry, rewet" rates. Collaborators at the EPA carried out other analyses on the same core samples, which included total C and N, as well as organic matter and denitrification rates.

Second Sampling. A second set of samples was collected in September 2010 to further investigate hypotheses that arose after interpreting the results from the April 2010 sampling. Only stream bank samples were collected at this time by inserting a hand-held core horizontally into the middle of the three layers of interest. This was repeated along the bank faces of the stream at 5 different locations. The same protocol as described above was carried out for all samples, except a third fresh sample was weighed into a Wheaton vial. This sample was left to air-dry for 7 days, like the "air-dry, rewet" soils, but after the 7 days, instead of rewetting the soils these "air-dry only" samples were extracted using 2.0 *M* KCl. Inorganic N levels were measured, and compared to the concentrations found in the "air-dry, rewet" samples in order to determine if any pulses found were more the result of air-drying or rewetting. Total C and N concentrations were determined for the stream-bank only samples by dry combustion elemental analysis. Loss on ignition (LOI) was also carried out on the samples to determine organic contents of the soils.

Microbial Analyses. A community level physiological profile (CLPP) of each stream bank soil sample was undertaken to better understand the diversity of microbial substrate use in the three layers present at BSR. Catabolic response was determined by the measurement of the

short-term respiration responses of soils after the addition of 15 different simple organic compounds. The substrates used in the assay were: D-glucose, citric acid, ascorbic acid, urea, asparagines, L-cysteine, glycine, lignin, pepsin, N-acetyl glucosamine, α-ketobutyric acid, malic acid, oxalic acid, tannin, and humic acid. These substrates represented a range of C sources, from labile to recalcitrant compounds.

Each carbon source was dissolved in deionized water and prepared at a concentration that would deliver 30 mg of C per g of soil water when 25 μL of each substrate was dispensed. The substrate solution was added directly to the soil samples once they had been incubated for ~5 days to reach an equilibrated WHC. Once the deep-well microplates contained both the soil sample and substrate solution they were placed face to face with a second microplate containing a detection gel. The two microplates were sealed together and incubated in a dark cabinet at room temperature for four 6-hour intervals. Immediately prior to sealing the two microplates to each other, and after each 6-hour interval, the CO_2-trap absorbance was measured at 570 nm using a microplate spectrophotometer. Quantities of CO_2 produced by each sample were calculated and reported as a CO_2 rate (μg CO_2-C g hr^{-1}). Catabolic responses were also standardized for each substrate in order to gain further information about the C sources microbes were utilizing most at different depths.

Catabolic responses were used to calculate catabolic evenness, a component of microbial functional diversity that reveals the uniformity of substrate use, by using the Simpson-Yule index: $E=1/\Sigma p_i^2$ (Degens et al., 2000a, b). Catabolic evenness (E) was calculated from the respiration response profiles, with p_i representing the respiration response to individual substrates (r_i) as a proportion of total respiration activity induced by all substrates (Σr_i) for each soil sample, i.e. $p_i=r_i/\Sigma r_i$ (Degens et al., 2000a). Since catabolic evenness is a measure of the

relative variability in the catabolic functions of the soil, it is a dimension-less unit. Using 15 different substrates (excluding the no-substrate control of water) the maximum achievable evenness, where all substrates respond equally, was 15 (Degens et al., 2000b). Richness, another component of diversity, was also determined, and defined as the number of substrates used by the microbes (Degens et al., 2000b).

The activities of seven extracellular enzymes were also determined for the stream bank soil samples according to methods described in Allison and Vitousek (2004) and Sinsabaugh et al. (1993). The hydrolytic enzymes cellobiohydrolase (CBH), β-glucosidase (BG), β-N-acetylglucosaminidase (NAG), leucine aminopeptidase (LA), and acid phosphatase (AP), in addition to the oxidative enzymes polyphenol oxidase (PPO) and peroxidase (PerO), were measured for absorbance using a microplate spectrophotometer.

Soil enzyme activity was measured on ~2 g wet weight subsamples. Soil samples were frozen prior to analysis, as is common in other studies (Allison and Vitousek, 2004; Keeler et al., 2008). Substrates were prepared as follows: AP: 5 mM pNP-phosphate; CBH: 2 mM pNP-cellobioside; BG: 5 mM pNP-β-glucopyranoside; NAG: 2 mM pNP-β-N-acetylglucosaminide; LA: 5 mM leucine p-nitroanilide; PPO and PerO: 5 mM L-dihydroxy-phenylalanine; all in 50 mM acetate buffer. Samples were combined with 60 mL of 50 mM, ph 5, acetate buffer, and homogenized in a blender for one minute. In a 2 mL Eppendorf tube 0.750 mL homogenate and 0.750 mL substrate were combined and shaken and incubated at 20°C for one to six hours. For every sample, three analytical replicates were prepared for each of the enzyme assays. Controls and blanks were included in order to account for any background absorbance of the homogenates or substrates. Following centrifugation, the supernatant of each sample was pipetted into a corresponding microplate well. 1.0 NaOH was also added to each well of the hydrolytic

enzymes to terminate the reaction and develop the color to be measured. The absorbances of the samples were read using a microplate spectrophotometer at 405 nm for the hydrolytic enzymes and 450 nm for the oxidative enzymes. Enzyme activity was measured as µmol of substrate converted per hour per gram soil organic matter.

The stoichiometric ratios of certain enzymatic activities were also analyzed in order to gain a better grasp of possible resource shifts in the different soil layers along the stream bank. The ratios BG:AP, BG:(NAG+LA), (NAG+LA):AP, BG:(PerO+PPO), and LA:NAG provided information regarding enzymatic C:P, C:N, N:P, labile C:recalcitrant C, and one form on labile N: another form of labile N.

Statistical Analysis and Data Treatment. Statistical analyzes were carried out using a combination of the softwares MINITAB 14 (Minitab Inc., USA) and SAS 9.1 (SAS Institute Inc., USA) for Windows. Proc Mixed of the SAS statistical software was used to examine whether mineralization rates and other chemical properties differed among depths and land positions, and if there was an interaction between depth and land position. When interactions (depth x land position) were observed , data were further analyzed by a one-way analysis of variance (ANOVA) If significance was found at the level of $\alpha \leq 0.05$ a Fisher's least-significance difference (LSD) multi-comparison test (with 95% confidence limits) was used to compare specific depths and/or land positions. Paired t-tests were also carried out to determine significance between fresh versus air-dried, rewet samples. All data were checked for normality and homoscedasticity, and log (or natural log, i.e. ln) transformed when necessary. Two outliers were removed based on semi-studentized residuals; if $|e^*| \geq 4$. MINITAB was also used to produce a correlation matrix to determine if there were any possible relationships between the different

data collected. Data were geographically managed and processed with the GIS software ArcMap 9.2 (ESRI Inc., California, USA).

CHAPTER 4

RESULTS

Soil Carbon and Nitrogen: Surface soils from the non-legacy upland and legacy zone had significantly higher total soil C and N than that found in their midlayer or bottom soils (Figure 4-1). They also both had higher C and N at the surface than the stream bank soils. In the

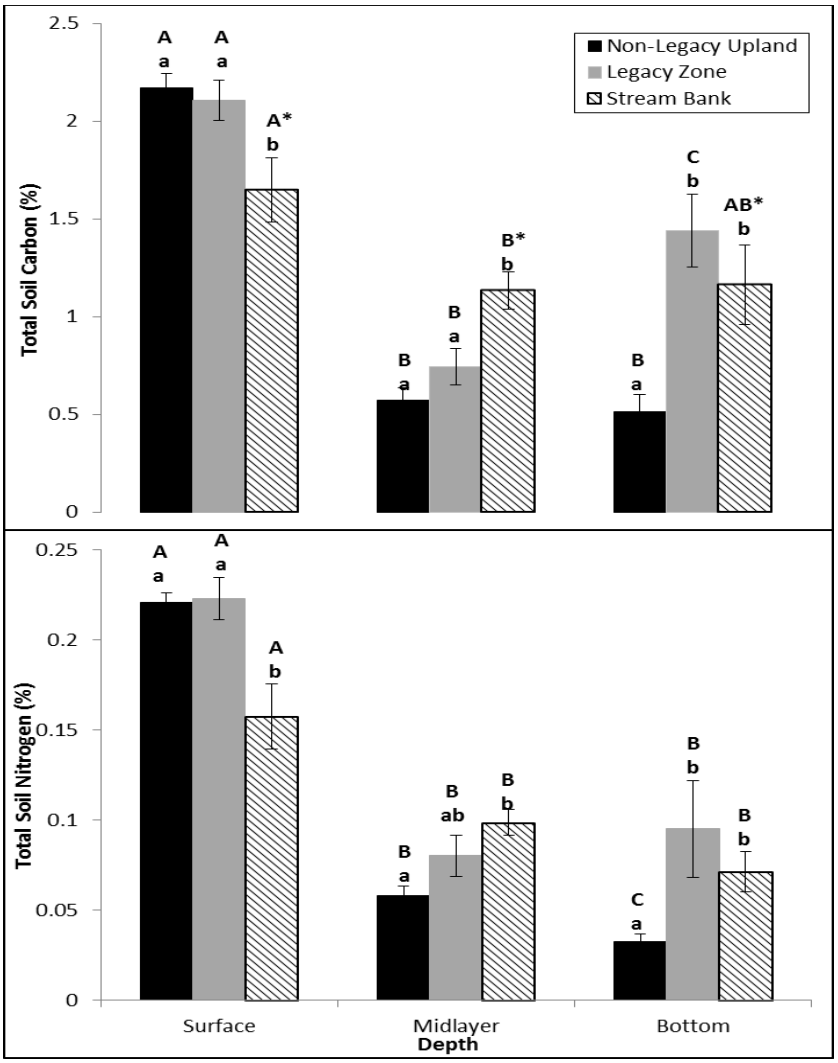

Figure **4-1**: Total soil carbon and total soil nitrogen expressed as averages across landscape positions and depths. Vertical bars denote one standard error of the mean. For a given depth, bars with different lowercase letters represent statistically significant ($p < 0.05$) differences between land positions. For a given land position, bars with different uppercase letters represent statistically significant ($p < 0.05$) differences with depth. At the stream bank landscape position depth was not statistically significant ($p = 0.071$) for total soil carbon, but a post-hoc test was still performed.

bottom soils, however, C and N were higher in the legacy zone and stream bank soils compared to the non-legacy upland, showing there was a large amount of C and N in the buried hydric soils. Stream bank soils also had significantly higher soil C and N compared to the non-legacy upland for the midlayer depth.

Soil Ammonium and Nitrate: Initial concentrations of NH_4^+ in the soils of BSR greatly dropped from the surface (4.91-8.17 mg/kg soil) to the midlayer (0.84-1.94 mg/kg soil) and bottom (0.78-2.90 mg/kg soil) depths for all three landscape positions – non-legacy upland, legacy zone, and stream bank (Table 4-1). The surface soils had significantly higher initial

		Depth		
		Surface	Midlayer	Bottom
			mg N/kg soil	
NH_4^+-N	Non-Legacy Upland	5.84 (0.52)^{a,A}	0.84 (0.11)^{a,B}	0.78 (0.17)^{a,B}
	Legacy Zone	8.17 (0.94)^{b,A}	1.28 (0.26)^{a,B}	2.90 (0.63)^{b,B}
	Stream Bank	4.91 (0.79)^{a,A}	1.94 (0.06)^{b,B}	2.14 (0.37)^{b,B}
NO_3^--N	Non-Legacy Upland	4.28 (0.54)	2.77 (0.58)	3.93 (0.62)
	Legacy Zone	4.10 (0.88)	1.77 (0.56)	2.09 (0.84)
	Stream Bank	4.23 (0.44)	2.83 (0.33)	4.97 (1.19)

Table **4-1**: Extractable ammonium (NH_4^+-N) and extractable nitrate (NO_3^--N) expressed as averages across landscape positions and depths. These represent initial concentrations (i.e. time zero levels) measured on fresh soils that were not incubated. Numbers in parentheses denote one standard error of the mean. For a given depth, values with different superscript lowercase letters represent statistically significant ($p < 0.05$) differences between land positions. For a given land position, values with different superscript uppercase letters represent statistically significant ($p < 0.05$) differences with depth. Initial NO_3^- concentrations were not statistically significant for any values.

NH_4^+-N concentrations than either the midlayer or bottom soils, regardless of landscape position. The two deeper depths, however, had similar initial NH_4^+-N in all landscape positions.

There was more variation in NH_4^+-N when concentrations were compared across landscape positions. Surface soils in the legacy zone had significantly higher concentrations

when compared to the surface soils found in the non-legacy upland or stream bank position. In the midlayer this changed – the stream bank had higher NH_4^+-N concentrations than the non-legacy upland or legacy zone. Both the legacy zone and stream bank bottom soils had significantly higher NH_4^+-N concentrations than the bottom soils of the non-legacy upland. There were no zones of naturally low NO_3^--N at the BSR site since there were no significant differences in NO_3^--N concentrations across landscape position or depth.

Potential Net Nitrification: Potential net nitrification rates varied across both landscape position and depth. Potential net nitrification rates were significantly lower in the midlayer and bottom soils of the non-legacy upland and legacy zone as compared to the surface soils of the two landscape positions (Figure 4-2). Stream bank soils had significantly lower nitrification

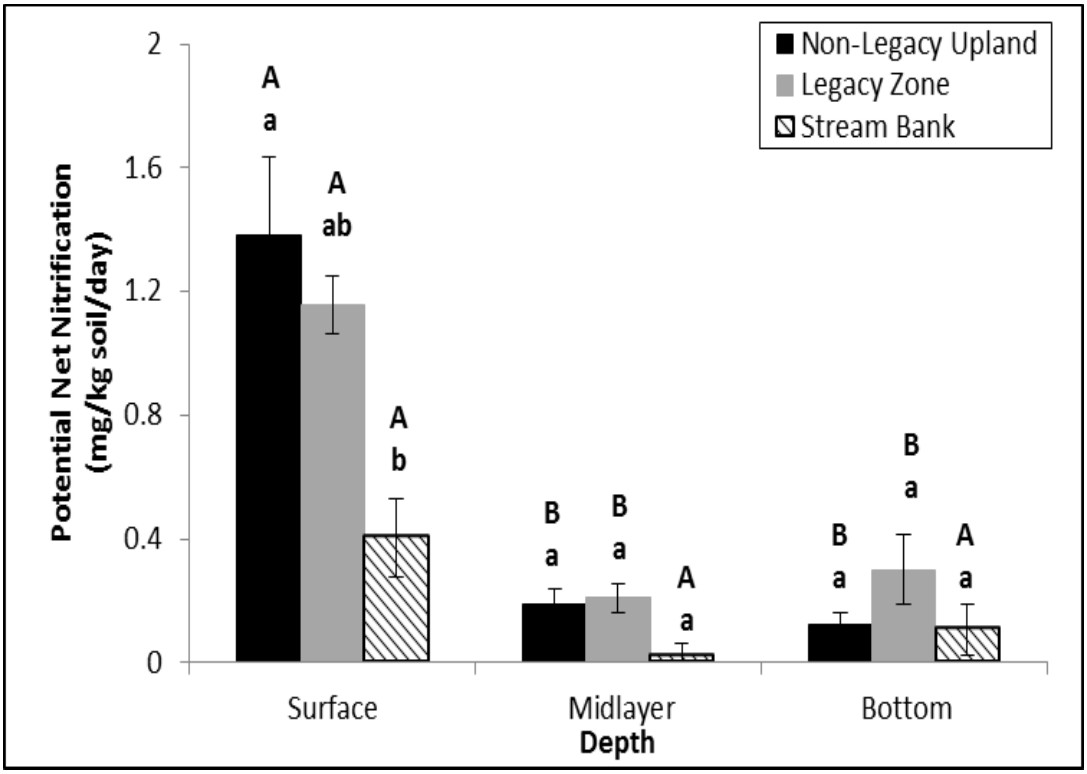

Figure **4-2**: Potential net nitrification rates expressed as averages across landscape positions and depths. Vertical bars denote one standard error of the mean. For a given depth, bars with different lowercase letters represent statistically significant ($p < 0.05$) differences between land positions. For a given land position, bars with different uppercase letters represent statistically significant ($p < 0.05$) differences with depth.

rates in the surface when compared to the positions farther from the stream. In addition, net nitrification rates did not differ across the three depths in stream bank soils. If net nitrification takes place near the stream, it may occur at the same rate regardless of depth.

***Potential Net Ammonification*:** Potential net ammonification rates were negative for all depths and landscape positions (Figure 4-3). Surface soils had the most negative rates for all

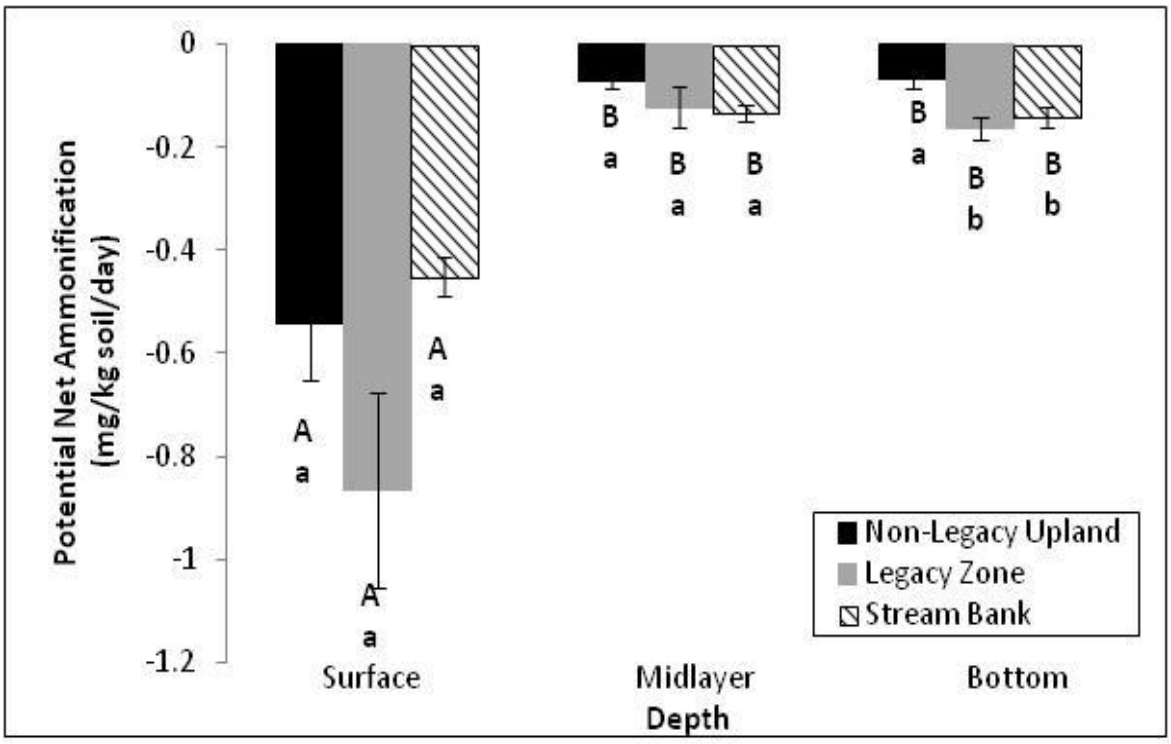

Figure **4-3**: Potential net ammonification rates expressed as averages across landscape positions and depths. Vertical bars denote one standard error of the mean. For a given depth, bars with different lowercase letters represent statistically significant ($p < 0.05$) differences between land positions. For a given land position, bars with different uppercase letters represent statistically significant ($p < 0.05$) differences with depth.

landscape positions. These ammonification rates were significantly different than soils from the midlayer and bottom soils, regardless of landscape position. Only in the bottom soils did landscape position affect net ammonification rates. Soils from both the legacy zone and stream bank positions had significantly higher negative rates than that found in the non-legacy upland soils at depth.

***Potential C Mineralization*:** Potential C mineralization was different across depths and land positions (Figure 4-4). Surface soils in both the non-legacy upland and legacy zone had

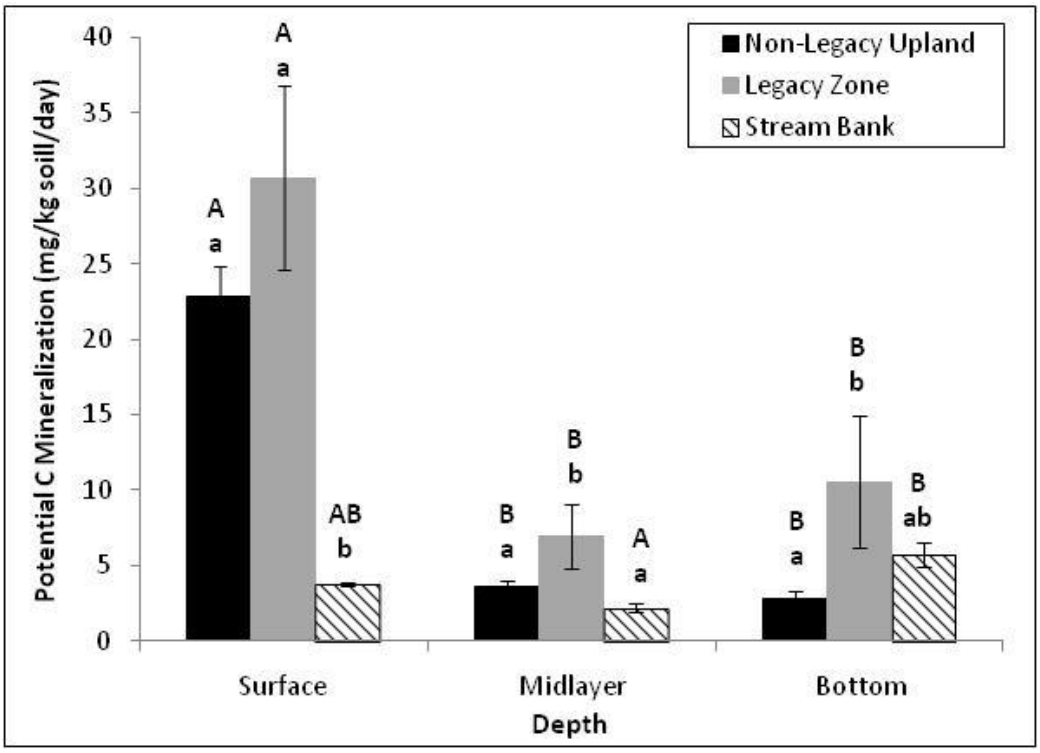

Figure **4-4**: Potential carbon mineralization rates expressed as averages across landscape positions and depths. Vertical bars denote one standard error of the mean. For a given depth, bars with different lowercase letters represent statistically significant ($p < 0.05$) differences between land positions. For a given land position, bars with different uppercase letters represent statistically significant ($p < 0.05$) differences with depth.

significantly higher mineralization rates (mgC/kg soil/day) than those found in the two lower depths. While C mineralization rates were similar at the surface for the non-legacy upland and legacy zone, in the midlayer and bottom soils the legacy zone samples were significantly higher than the non-legacy uplands. This trend mimicked the total soil C and N data in the bottom soils where the legacy zone soils had significantly higher soil C and N than non-legacy upland soils.

For stream bank samples at the surface C mineralization was significantly lower than in the other two landscape positions. In the midlayer and bottom soils, however, stream bank soils were not as drastically different across landscape positions. Non-legacy upland soils and stream

bank soils in the midlayer had similar mineralization rates. While, at the bottom depth the mineralization of C in stream bank soils was not significantly different than either of the other two landscape positions.

Surface soils had high C mineralization rates, but bottom soils in the legacy zone and stream bank samples also showed some signs of enhanced microbial C use. The bottom hydric soils of the stream bank had higher mineralization rates than those collected from the midlayer depth. C mineralization rates in the legacy zone were similar for both the midlayer and bottom soils, however, these rates were significantly lower than the rates found at the surface soils of the legacy zone.

Community Level Physiological Profile: The community level physiological profile for microbes at each depth was only determined for stream bank samples, where bottom soils were known to be hydric soils and midlayer soils were known to consist of legacy sediment. Overall, catabolic response was greatest in the surface soils of the stream bank, regardless of substrate type (Figure 4-5). Hydric soils had significantly lower responses to added substrates than surface soils, with the midlayer soils having an intermediate response. At the surface, where the highest overall respiration occurred, microbes appeared to respond more to labile carbon, like D-glucose, and amino acids, such as glycine and asparagine. Only for the D-glucose substrate, however, was there a significant difference in standardized catabolic response. The surface soils had a significantly higher standardized catabolic response to the added D-glucose than the midlayer and bottom soils. At depth there was no statistically significant difference in the use of substrates. Observationally it appeared that in the hydric soil and the subsurface legacy sediments, microbes responded more when presented with recalcitrant carbon sources, like tannin and humic acid, which consist of more complex compounds. Increased sampling may

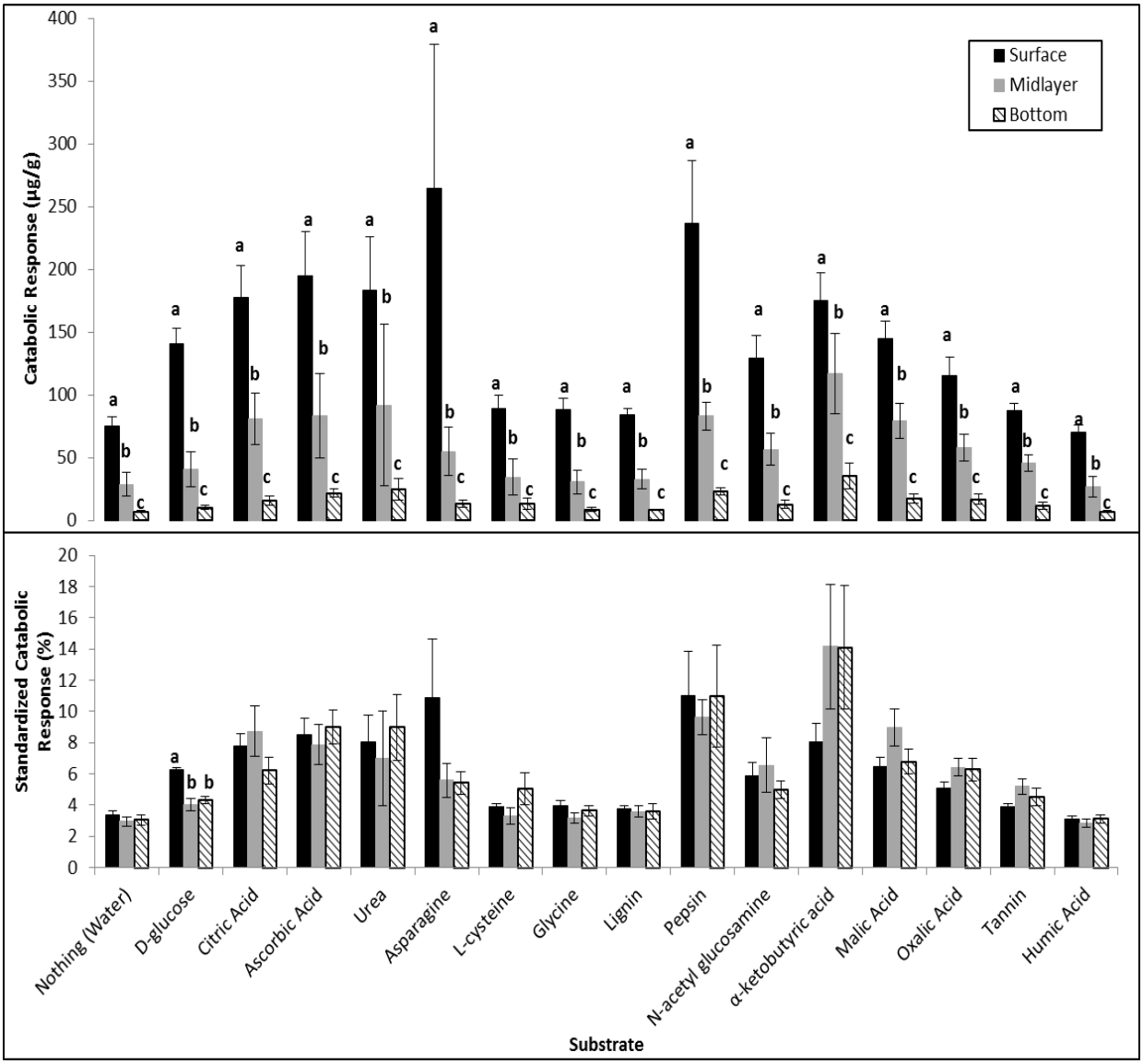

Figure **4-5**: Catabolic response and standardized catabolic response as averages for stream bank samples across three depths. Vertical bars denote one standard error of the mean. For a given substrate, bars with different lowercase letters represent statistically significant ($p < 0.05$) differences between depths. Only the standardized ratio of D-glucose was statistically significant ($p < 0.05$) with depth as depicted by the different lowercase letters over the bars for D-glucose.

have confirmed this observation statistically. Therefore, the great shift from high to low catabolic activity cannot be explained by any shift in the utilization of labile versus recalcitrant carbon substrates at different depths.

There were catabolic responses to all the substrates tested in this project, showing that

richness did not differ between the soils. Evenness, the variability of substrate use across the range of substrates tested, ranged among the stream bank soils at BSR from 8.8 to 14.8 at BSR. The differences in catabolic evenness at the three depths of interest, though, were not significant (p=0.694).

***Extracellular Enzyme Activity*:** Enzymatic activity (µmol/h gOM) was significantly higher in the surface soils along the stream bank than in the samples collected in the midlayer and bottom soils (Figure 4-6). The activity levels in the two lower soil layers, however, were not

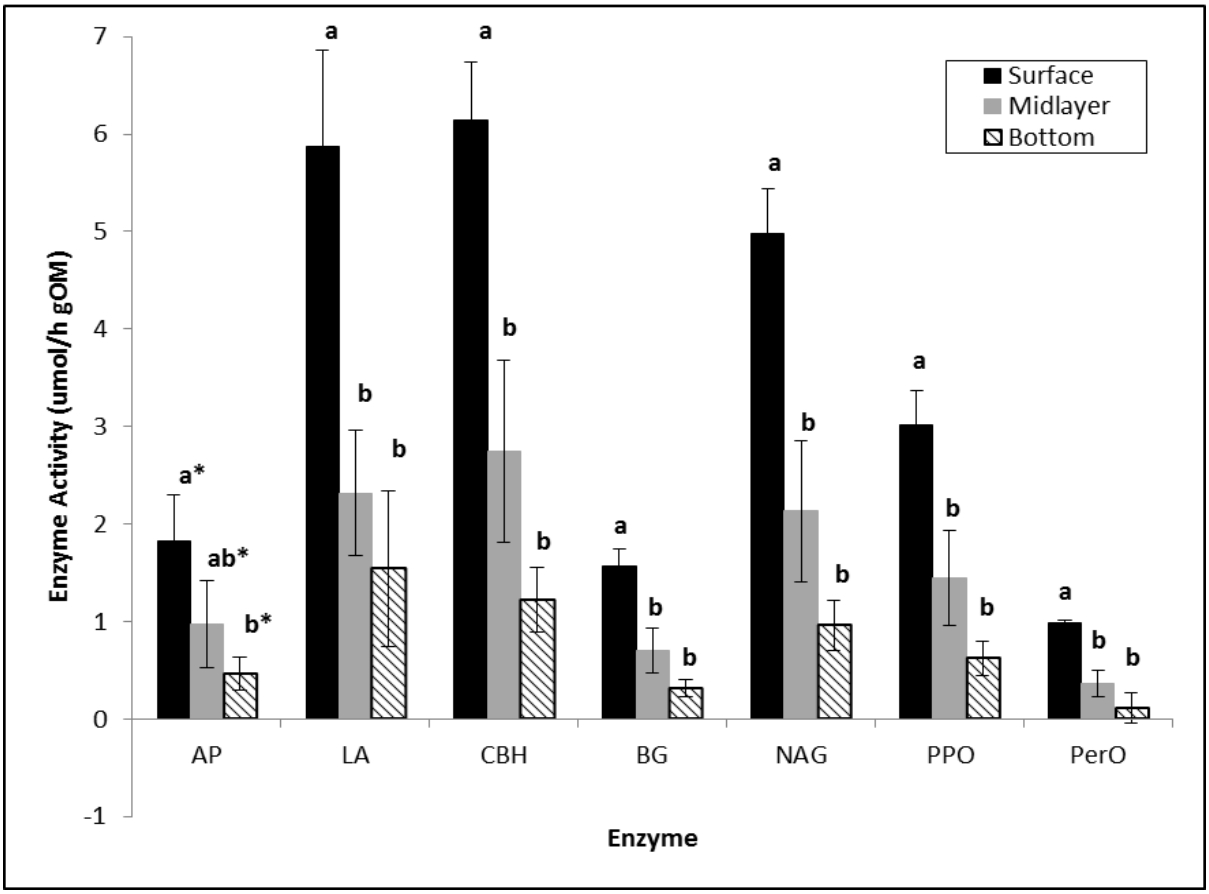

Figure **4-6**: Enzyme activity expressed as averages for stream bank samples across three depths. Vertical bars denote one standard error of the mean. For a given enzyme, bars with different lowercase letters represent statistically significant ($p < 0.05$) differences between depths. For AP (acid phosphatase) depth was not statistically significant ($p = 0.077$), but a post-hoc test was still performed. The results of the Fisher's least-significance difference (LSD) test for the AP enzyme are noted by the asterisks next to the letters.

significantly different than one another. The only enzymatic ratio that showed significance was BG:(PerO+PPO) (Figure 4-7). It was found that the bottom, hydric layer had a significantly

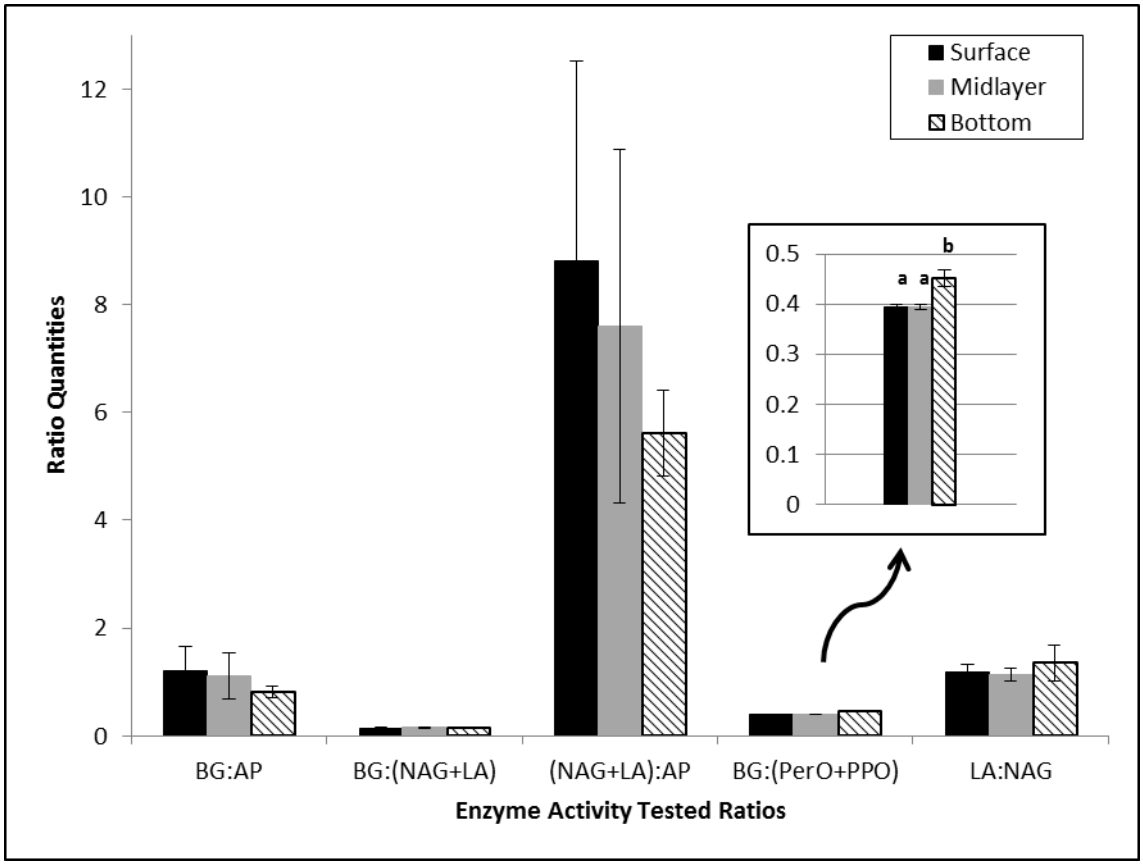

Figure **4-7**: Ratios of enzyme activities to one another for stream bank samples across three depths. Vertical bars denote one standard error of the mean. Only the ratio of BG:(PerO+PPO) was statistically significant ($p < 0.05$) with depth. This is depicted by the different lowercase letters over the bars in the enlarged box view of BG:(PerO+PPO).

higher ratio of BG:(PerO+PPO) than the two upper soil layers. This ratio represents the relative abundance of microbes attacking labile versus recalcitrant C compounds. There was an increase in BG:(PerO+PPO) in the hydric soil, which shows that there is less recalcitrant C enzyme activity in the long-buried layer. This finding does not support the above hypothesis that hydric soils are probably responding more to recalcitrant C substrates than labile sources.

Pulse Data Following Rewetting of a Dry Soil: A paired t-test was used to determine if the difference between air-dried, rewetted soils and fresh soils was significant (Table 4-2). The

		Depth					
		Surface		Midlayer		Bottom	
		Mean Difference	*p-value*	*Mean Difference*	*p-value*	*Mean Difference*	*p-value*
		mg N/kg soil/day					
NH_4^+-N	Non-Legacy Upland	0.60	0.000	0.09	0.000	0.06	0.003
	Legacy Zone	0.76	0.005	0.13	0.018	0.14	0.000
	Stream Bank	0.00	0.002	0.09	0.006	0.09	0.011
NO_3^--N	Non-Legacy Upland	-0.14	0.520	-0.04	0.420	-0.07	0.099
	Legacy Zone	-0.10	0.337	-0.04	0.392	-0.21	0.094
	Stream Bank	0.07	0.612	0.07	0.205	0.06	0.508

Table **4-2**: Paired t-tests comparing fresh soil and air-dried, rewetted soil across all landscape positions and depths for net ammonification (NH_4^+-N) and net nitrification (NO_3^--N). Mean differences represent the nutrient pulses that follow the rewetting of a dry soil. Positive mean differences indicate a flush in the corresponding process, while negative mean differences indicate there was no flush in the corresponding process following rewetting. Statistical significance was set as $p \leq 0.05$.

potential net nitrification rates in fresh versus air-dried, then rewet, soils were not significantly different between the two conditions at depth for any of the three landscapes (Figure 4-8). The

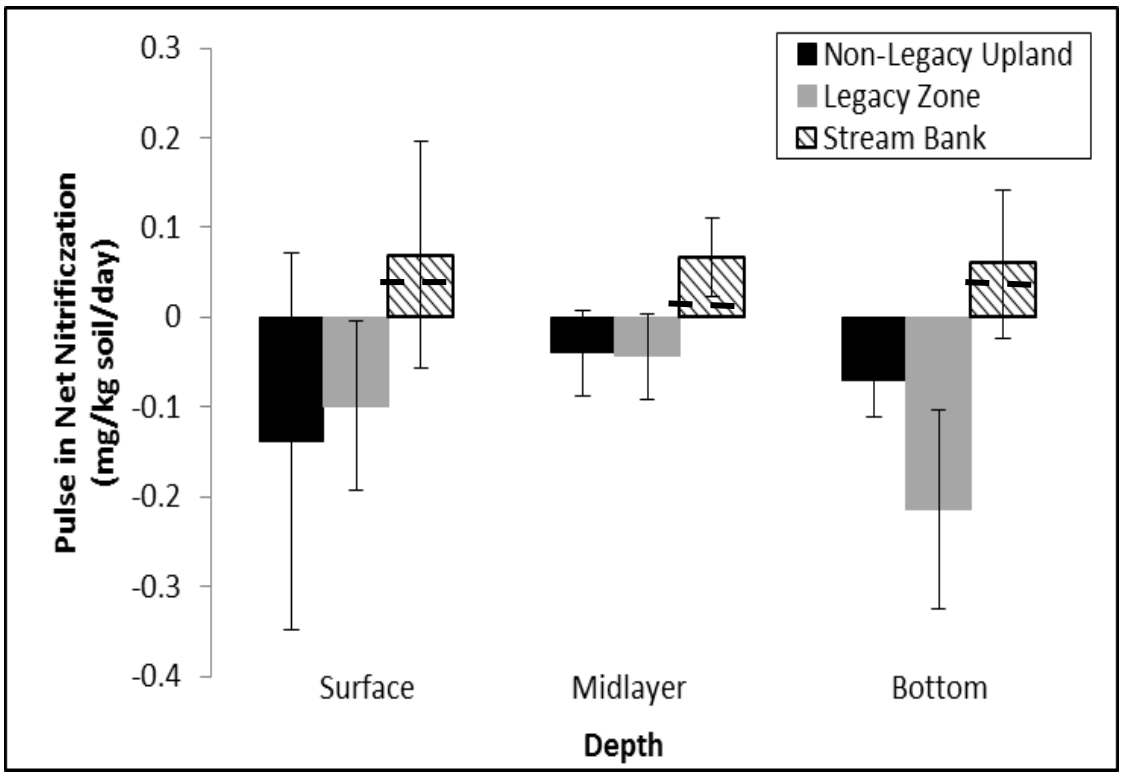

Figure **4-8**: Pulses in net nitrification following rewetting of a dry soil expressed as averages across landscape positions and depths. Vertical bars denote one standard error of the mean. Pulses were not statistically significant across the three landscape positions and three depths. Since there was no significance, no letters are listed. The dashed lines drawn over the stream bank nitrification pulses depict the contribution of air-drying (amount below the dashed line) versus rewetting (above the dashed line) to the overall drying/rewetting effect.

trends in the calculated pulses were not significantly different, either. The magnitude changes in these pulses were quite small, so it is unclear whether there is any difference in NO_3^--N cycling following the rewetting of an air-dried soil; or, if changes in gross nitrification and gross NO_3^--N immobilization are cycling in such a way to cancel any NO_3^--N gains or losses.

At each of the depths sampled, landscape positions were found not to significantly affect the pulse of net ammonification that followed the rewetting of an air-dried soil (Figure 4-9).

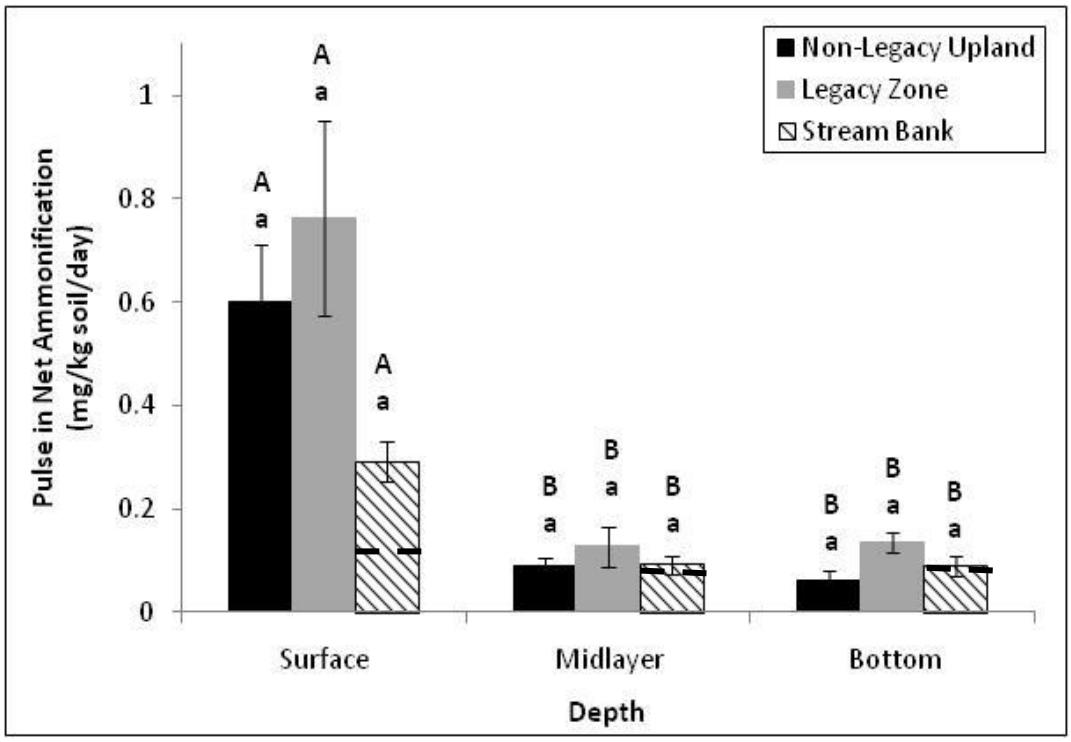

Figure **4-9**: Pulses in net ammonification following rewetting of a dry soil expressed as averages across landscape positions and depths. Vertical bars denote one standard error of the mean. For a given depth, bars with different lowercase letters represent statistically significant ($p < 0.05$) differences between land positions. For a given land position, bars with different uppercase letters represent statistically significant ($p < 0.05$) differences with depth. The dashed lines drawn over the stream bank ammonification pulses depict the contribution of air-drying (amount below the dashed line) versus rewetting (above the dashed line) to the overall drying-rewetting effect.

There was a trend in pulse rates with depth, however. Surface soils showed significantly higher pulses in net ammonification than that found in the midlayer or bottom soils, regardless of landscape position. Analysis of the contribution of drying vs. rewetting to the increased pulse in net ammonification revealed that rewetting air-dried soils influenced the magnitude of the ammonification pulse the most.

Correlations: Many relationships between certain soil characteristics were expected, like that between total percentage N and total percentage C, which had $R^2 = 0.73$ ($p = 0.000$), 0.72 ($p = 0.000$), and 0.94 ($p = 0.000$) for the surface, midlayer, and bottom layer, respectively. Of interest, however, was that soil moisture was found to be negatively correlated with all the

exoenzymes utilized for this project. Correlation coefficients were lowest for AP (*R2* = 0.46, p = 0.006) and LA (*R2* = 42 0.54, p = 0.002). They were highest for PerO (*R2* = 0.64, p = 0.000), PPO (*R2* = 0.68, p = 0.000), BG (*R2* = 0.68, p = 0.000), CBH (*R2* = 0.69, p = 0.000), and NAG (*R2* = 0.69, p = 0.000). Soil organic C, total C, total N, and net nitrification were not correlated with any of the enzymes, however.

CHAPTER 5

DISCUSSION

The main focus of this study was to analyze the controls on nitrification rates at BSR. There are many controls on nitrification that interact on the long-term and short-term scale. This study concentrated on three controls in particular – NH_4^+, C quality and quantity, and climate. As depicted in Figure 5-1, a change in NH_4^+-N concentrations would directly affect the

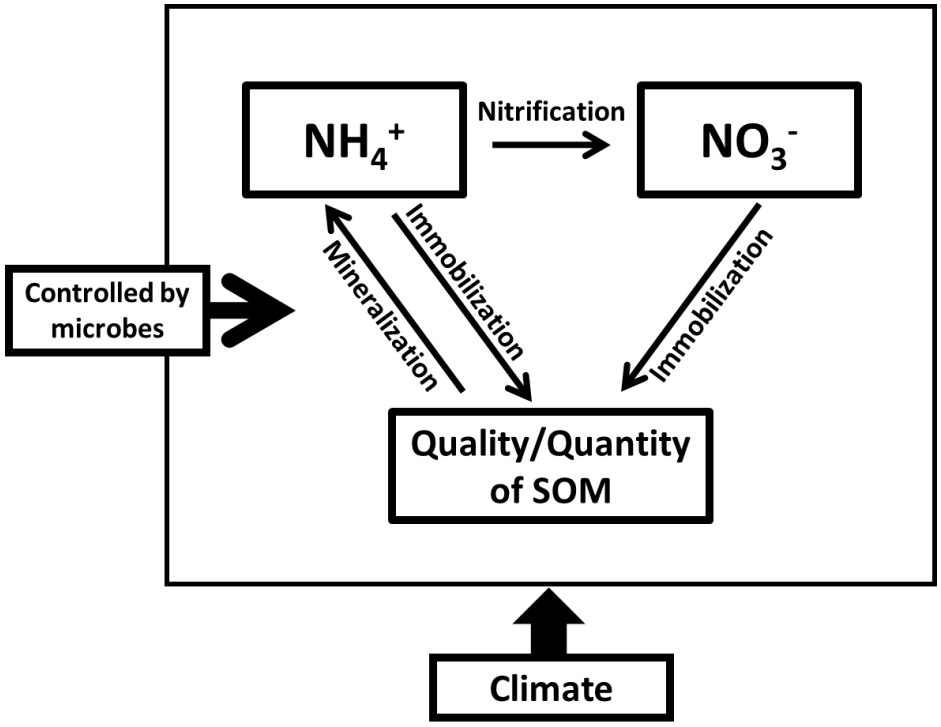

Figure **5-1**: Model showing possible controls on nitrification rates. The interactions between ammonium (NH_4^+-N), nitrate (NO_3^--N), and the quality and quantity of soil organic matter (SOM) may change in different climatic conditions.

nitrification process. The size and quality of C and N pools are impacted by soil organic matter (SOM). This in turn can impact microbes, which control the system's rates of mineralization and immobilization. External to these processes is climate, which has the potential to change the interactions within the N cycle, and thus affect nitrification.

The N cycle has many components that affect the production and immobilization of NO_3^--N. The high C concentrations in the surface soils of BSR could promote heterotrophic activity. Heterotrophic soil microorganisms are capable of producing a wide variety of extracellular enzymes that can convert organic N into NH_4^+-N. Mineralization can also occur when heterotrophs assimilate low C:N substrates. Increased pools of NH_4^+-N due to mineralization would be available to the nitrifier populations for conversion to NO_3^--N. Depending on the C:N ratio of available substrates heterotrophic microorganisms may need to assimilate more N, leading to the immobilization of NO_3^--N. There is mixed evidence, however, for high rates of NO_3^--N immobilization at BSR.

Factors influencing nitrification revealed interesting relationships among the three landscape positions (non-legacy upland, legacy zone, and stream bank) and three depths (surface, midlayer, and bottom) at BSR. Patterns found across depth were much stronger than those found across landscape positions. However, there were some significant differences in concentrations and rates among the three landscape positions. Net nitrification rates in the legacy zone and non-legacy uplands were not found to be significantly different, refuting the original hypothesis that rates would be greater in the legacy zone due to a higher initial pool of NH_4^+-N. Of greatest interest, however, was the landscape pattern which showed that the non-legacy upland soils were significantly different than the stream bank soils for both total soil C and N at all three depths. This finding suggested that there was something inherently different between the two landscape positions; non-legacy upland soils lacked hydric layers, which were present in the stream bank soils. The differences in concentrations and rates across depth further revealed the distinctiveness of the bottom hydric soils, which showed patterns dissimilar to the surface soils. Thus, assessing depth patterns will allow nitrification processes at BSR to be better understood.

Surface Soils: The high concentrations of NH_4^+-N and C in the surface soils may have promoted the high microbial activity found in this layer. Despite this, nitrifiers appeared to compete well for NH_4^+-N resulting in high net nitrification rates at the surface. The high net nitrification rates imply that immobilization of NO_3^--N did not dominate the system at the surface, otherwise net nitrification would have been negative. One interpretation that is parsimonious with our results is that the surface layers were N-rich, and thus NH_4^+-N was available for nitrification, and high gross NO_3^--N immobilization did not lead to net NO_3^--N immobilization, even though there was high C availability. This pattern is analogous to N saturation, in which N inputs exceed plant and microbial capacity to effectively use or retain N (Bernot and Dodds, 2005). The N not incorporated into growth processes can then be released from the system by means of microbial processes and N loss to ground and surface water.

Increased rates of soil nitrification are characteristic of N saturated systems (Hanson et al., 1994). Prior land-use history can play a significant role in current landscape responses to N inputs (Aber et al., 1998). Indeed, according to Aber et al. (1998), land use is more important in determining current NO_3^- leaching losses in the northeastern U.S. than current or total accumulated N deposition. The uplands at BSR were once used as agricultural fields, and now largely serve as pastures. High inputs of N in these uplands due to grazing and cropping may have caused N saturation in the surface soils. Past land uses may have subjected the surface soils of the legacy zone and stream bank to high inputs of N via erosion and/or runoff from the non-legacy upland areas, and thus could also exhibit effects of N saturation.

Midlayer Soils: The midlayer had significantly lower rates of net nitrification compared to the surface soils, but had similar rates compared to the bottom soils. The midlayer soils also had lower total soil C and N than both the surface soils and bottom soils, which may explain its

low net nitrification rates. Unlike the surface soils, the midlayer soils had low C mineralization rates, as well. Low C mineralization rates would suggest that N immobilization rates would also be low. However, both net nitrification and net ammonification rates were found to be low in the midlayer soils, which implies that the low net N mineralization rates are probably due to low gross inorganic N production, coupled with low immobilization. Thus, overall, the midlayer soils seem to be nutrient and C poor, with low C mineralization, low net N cycling rates, and low pool sizes. We suggest that this layer is likely not a dynamic N filter, but rather acts to transfer N produced in the above surface layer to deeper soils.

Hydric Soils: We expected there would be net NO_3^--N immobilization in the hydric soil due to its high C concentrations, and the higher potential C mineralization that was found in the hydric soil relative to the other deep soils lacking the hydric layer. This was not the case, however, as there was little evidence for high NO_3^--N immobilization. There are several possibilities as to why the hydric soils did not exhibit net NO_3^--N immobilization. It was expected that the small increase in respiration found in the hydric soil should have acted as fuel for the microbes, stimulating NO_3^--N immobilization. Perhaps this increase in respiration was not large enough to induce gross immobilization of the NO_3^--N. High gross nitrification rates could also impact net nitrification rates. If gross nitrification rates were high one would expect high NH_4^+-N consumption. However, NH_4^+-N consumption in the hydric soil was low compared to the surface soils, suggesting that high gross nitrification rates were not responsible for the low net nitrification found in the hydric soil. N saturation in the hydric soils could also account for the absence of NO_3^--N immobilization at depth. If there was a constant flow of high-N water through the hydric layer it could be plausible that NO_3^--N sinks were already saturated. Unlike the surface soils, however, the hydric soils showed low net nitrification rates, which challenges

the idea that the hydric soil was N saturated. Given the low net nitrification potential at depth, the high initial NO_3^--N concentrations in the hydric soils likely flowed in from another source, as the low oxygen conditions in the hydric soils is expected to inhibit nitrifiers (Pierzynski et al., 2005). The interconnectedness of immobilization and mineralization processes makes it difficult to parse out exactly why NO_3^--N immobilization did not occur in the hydric soils as expected. Future ^{15}N tracer studies of BSR soils will allow more insight into the partitioning of such processes.

The generally low microbial activity in the hydric soils, too, may account for the poor NO_3^--N filtration seen in the bottom soils. Originally, it was hypothesized for research question 4 that the long-buried hydric soil would be biogeochemically active, with the ability to support high levels of microbial activity. Catabolic responses, however, showed low microbial activity at depth, suggesting that the hydric soil was much less biogeochemically active than predicted. When stream bank samples were exposed to a range of labile and recalcitrant C substrates hydric soils responded weakly to all C substrates. The response of the buried soils was significantly lower than both the surface and midlayer soils of the stream bank. These results suggest that the low microbial activity found in the long-buried hydric soils of the stream bank was not due to a lack of a specific substrate.

Since the stream bank soils at BSR were not limited by different forms of C, the response of existing enzymes in the soil to substrate saturation was investigated to determine if a different element was more limiting in the system. AP, a crucial enzyme involved in the transformation of organic P, had rather low activity levels across all three depths. Increases in AP activity have been interpreted as reflecting an increase in P demand by soil microbes (Currey et al., 2010). Thus, given the low activities of AP along the stream banks of BSR, a possible explanation could

be that the soil microbial community is not P-limited. High levels of P have previously been found at BSR (Walter and Merritts, 2008), suggesting that P limitation at the site is unlikely.

The activities of exoenzymes are frequently linked to rates of microbial metabolism and biogeochemical processes (Sinsabaugh et al., 2009). For this reason they can be used as indicators of microbial nutrient demand in the soils. This demand is determined by the stoichiometric activity ratios of enzymes that catalyze terminal reactions which can lead to the production of assimilable products from elemental C, N, and P sources (Sinsabaugh et al., 2009). The only shift in enzymatic ratios was found in BG:(PerO+PPO), which increased in the hydric soil. This suggests that there was less recalcitrant C enzyme activity in the long-buried layer, and that microbes were attacking more labile C at depth. This finding was not consistent with the observational hypothesis in which it was expected that hydric soils would respond more to recalcitrant C substrates.

External Controls on Nitrification: To address research question 3 it was hypothesized that potential net nitrification would increase in the soil at all three landscape positions and depths at BSR when rewetted after a drought period. This phenomenon did not occur as expected. When comparing nitrification rates between fresh samples and air-dried, rewet samples, no significance was found. And, while stream bank samples at all depths appear to have positive NO_3^--N pulses following rewetting and the other landscape positions appear to lack these flushes, samples were not found to be significantly different. These results suggest that NO_3^--N flushing did not occur in any soils of BSR. A balance between the two opposing transformations – gross nitrification and gross NO_3^--N immobilization – could explain why the expected large pulse in NO_3^--N did not occur. Incubation methods could have also affected the results. The net nitrification rates for the fresh soils and the air-dry, rewet soils were based on

different incubation times. The rates for fresh soils were measured over a 7 day period. For air-dried, rewet soils, however, rates were measured over a 14 day period. Soils were air-dried for 7 days, then after rewetting, a laboratory incubation of 7 days was carried-out. Thus, rate calculations were based on two different lengths of time.

It was expected that rewetting would contribute more to the pulses because air-drying generally decreases microbial biomass (Sparling and Ross, 1998). However, rewetting the air-dried soil contributed less to the pulse in nitrification than the drying phase, suggesting that the absence of NO_3^--N flushing could be due to slow or suppressed recovery of microbial communities following rewetting after a drought. The duration of drying and rewetting is of importance for NO_3^--N pulses. Short drying periods followed by extended rewetting periods have been known to result in greater NO_3^- flushes (Borken and Matzner, 2009). The population of soil microorganisms can be influenced by the duration of drought conditions, which can then affect populations upon rewetting. Reductions in microbial activity could arise due to time-dependent decreases in overall microorganism populations (Schimel et al., 1999). Microbial populations present in the soil may have been adapted to such dry-wet cycles, and thus could better equilibrate during and after such stresses. A shift in bacterial community composition could also affect the magnitude of the NO_3^--N pulse following the rewetting of a dry soil. If drying and rewetting did not affect the bacterial community then it could have been hypothesized that the microbes were adapted to stress imposed by the dry-wet cycle (Borken and Matzner, 2009). This study, however, cannot comment on the microbial community changes following the rewetting of air-dried soils.

The difference in net ammonification rates between fresh and air-dried, rewet soils were all significant, which resulted in significant pulses of NH_4^+-N at all depths and landscape

positions. The ammonification pulse differences were of a greater magnitude than that of the nitrification pulses, and thus contributed the most to the change in net N mineralization after rewetting. The positive pulse in net ammonification following rewetting suggests that more organic N was available for mineralization to NH_4^+-N, and that the NH_4^+-N pool was large enough that it could have promoted NH_4^+-N oxidation to NO_3^--N without utilizing all the free NH_4^+-N in the soil solution. The pulses of NH_4^+-N following rewetting also suggest that larger pulses are generated in the surface soils, not the soils at depth. These pulses at the surface are not significantly different among the three landscape positions, though, which means drought-induced NH_4^+-N pulsing can occur along the whole landscape gradient at BSR. The mechanism that contributed most to these large pulses at the surfaces came mostly from the rewetting of the soil. In the two lower depths drought contributed the most to the NH_4^+-N pulses. This may mean that microbes were not recovering from the drought as quickly as was expected.

CHAPTER 6:

CONCLUSIONS

Clear patterns of N cycling soils consisting of legacy sediments were found at Big Spring Run. Potential net nitrification rates were found to be high in non-legacy upland and legacy zone soils. Stream bank soils tended to have the lowest nitrification rates of the three landscape positions utilized for this project. Nitrification rates were naturally higher in the surface layers at BSR as compared to the midlayer and bottom soils, regardless of landscape position. Total soil C and N were also highest in the surface soils, which may account for the correspondingly high nitrification rates at the surface. The question as to whether increased turnover of N and C at BSR is due to the cycling of nutrients stored *in situ* or due to the cycling of N and C transported to the area via erosion, runoff, etc. still needs to be investigated, however.

Riparian NO_3^--N attenuation depends on the ability of riparian vegetation and microbial communities to process N moving in surface runoff and groundwater flow (Groffman et al., 2002). There are several factors that can affect the NO_3^--N removal function of such systems, however. Altered hydrologic flow paths along the landscape can affect the processing of NO_3^- in soils. Highly incised channels and reduced infiltration can reduce near-stream groundwater levels, which can greatly affect the interaction of NO_3^--N with soils, impacting the immobilization or denitrification of NO_3^--N. Low water tables and N enrichment could increase nitrification rates, however. Recent work on deeply buried organic layers that are several thousand years old suggest that they can deplete NO_3^--N along deep groundwater flow paths (Hill, 2010; Gurwick et al., 2008). Gurwick et al. (2008a) stated that C availability in such buried soils may be determined by the abundance and quality of organic matter at the time of burial, instead of by the duration since burial. This implies that microbial activity in the buried

soils is largely disconnected from surface ecosystems (Gurwick et al., 2008a). However, lowered water tables could lead to a depletion of organic matter in the soil due to increased aerobic conditions, limiting immobilization (Groffman et al., 2002).

Substantial differences in the ability of microbial communities to metabolize a range of C substrates were observed in the stream bank soils of BSR at different depths. Microbial activity was especially low in the bottom soils. Even when presented with large supplies of different C sources hydric soils showed minimal catabolic response. This suggests that the long-buried hydric soil has a low potential to immobilize NO_3^-. Determining how legacy sediments and buried hydric soils process N under different hydrological flow regimes and patterns will help in better understanding why NO_3^- removal at depth was virtually absent.

In July of 2011, legacy sediments will be removed throughout a portion of the BSR watershed, which will expose the buried wetlands and reconnect the original floodplain hydrology of the site. This restoration effort represents a unique opportunity to assess the effects of watershed restoration on ecological function. It will be important to investigate whether microbial activity will increase in the hydric soil once it is exposed. If so, the time to recovery could have implications for the future management of similar sites.

The determination of net N mineralization can provide useful information regarding the potential availability of N for plants or the possibility of N loss via leaching (Borken and Matzner, 2009). The water flux within the soil and the uptake of N by plants largely dictate the amount of N loss that can occur following the drying and rewetting of soils. The annual rates of net N mineralization or inorganic N fluxes, such as NO_3^- leaching, have been observed to decrease with increasing duration and intensity of drought conditions (Emmett et al., 2004; Borken and Matzner, 2009). The pulse of net N mineralization following the rewetting of dry

soils can be relatively small or insignificant on an annual scale. After prolonged summer droughts, and subsequent wetting, N mineralization rates will decrease overall in the ecosystem. Short wetting pulses, if able to release large NO_3^- pulses in the future, will not generally be able to compensate for the low mineralization rates experienced during drought periods (Borken and Matzner, 2009). Increasing precipitation during the growing season, however, has the potential to enhance leaching losses of inorganic N in ecosystems characterized as N-rich (Borken and Matzner, 2009). Frequent precipitation increases soil moisture, which in turn can promote the decomposition of soil organic matter.

The impact of drying-rewetting cycles is of great interest in light of the predicted effects of climate change on weather patterns. Accelerated transport from landscapes to streams, and the reduction in retention capacities for nutrients like NO_3^-, could lead to the amplification of contaminant pulses during climatic extremes (Kaushal et al., 2010). Climate change models predict an increase in the variability of precipitation and hydrologic events, as well as an increase in the intensity of extreme weather conditions (IPCC, 2007). While not found in this short-term study, large pulses of contaminant exports may be flushed during such extreme weather events in the future. These storm systems will likely increase in frequency and magnitude with future climate change.

To fully understand how contaminants, like NO_3^-, enter the waterways at BSR it was important to determine possible ways N could be moving on the landscape. Rewetting air-dried soils did not result in any significant increases in the pulse in net nitrification rates at any landscape position or depth. This was unexpected, as region wide NO_3^- flushing had already been observed (Kaushal et al., 2010). Such flushing was found in streams over a much longer

time scale, though. It is thus still uncertain how, or if, the NO_3^- in soils is entering nearby streams.

Little work has been completed on nutrient cycling in legacy sediments. The prevalence of legacy sediments across the mid-Atlantic region is only now being addressed. For this reason quantifying changes in N along legacy sediment impacted streams is necessary. A key result of this study shows that NO_3^- that is generated in surface soils is not being immobilized in the C-rich hydric soil. These buried hydric soils have low biological activity and low nitrification potential, which may be due to a lack of labile C. Low activity can decrease N transformations, which explains why the buried hydric soil had low denitrification rates and did not act as a strong NO_3^- immobilizer. Depending on how water moves at BSR, however, N may be affected differently. Thus, the results of the above project have led to the further questioning of how N moves through the landscape under different conditions. Future studies will explore how hydrological flowpaths impact contaminant pulses in, and between, the main layers at Big Spring Run, and how interactions change after restoration. This is a new and necessary step to take in order to better understand N movement in legacy sediments and how to reduce its entrance into downstream waterways.

APPENDIX

Methods, Materials, and Analytical Techniques

In this study, several analytical techniques were used to characterize the processing of N within collected soil samples. Samples for this project were collected at two separate times. Results found for the first set of samples revealed trends that were of interest, so a second set of samples were collected in order to test new hypotheses. Retention and release of N in its different forms following drying-rewetting events was explored. The microbial diversity in the three layers of interest at BSR were also determined, revealing differences in activity levels, as well as differences in microbial populations present within the soils.

Respiration rates as well as concentrations of NH_4^+ and NO_3^- were measured in fresh, dried, and rewetted soil samples. A differential gas analyzer, LiCor-7000 CO_2/H_2O Gas Analyzer, was used to measure CO_2 rates over a 7 day incubation period. Using a 2.0 *M* KCl extraction technique inorganic N ions were extracted and their concentration levels were determined on a microplate spectrophotometer. N in the form of NH_4^+ and NO_3^- ions were of particular concern because they are very mobile forms of N and are most likely to be lost to the environment (National Research Council, 1993). The microplate spectrophotometer was also utilized to ascertain community level physiological profiles and enzyme activities in bank-face samples. These analytical procedures provided information concerning the functional diversity of the microbes present at BSR and helped to identify possible rate limiting steps of decomposition and nutrient cycling at the site. Other techniques were used to gain further understanding of the soil samples, including gravimetric water content, and loss on ignition (LOI), which was used to determine a crude estimate of the organic content for each sample.

I. Analyses of April & September 2010 Samples

Sample Collection. Soil samples along Big Spring Run were collected in April 2010 for four days – April 5th through 8th. An EPA drill team collected 29 soil cores in 4.7 cm diameter plastic tubes down to refusal using a hydraulic corer. Core locations were geospatially referenced with the use of a GPS system, and are shown in Fig.3-1. The sampling of soils was random across the landscape, but included cores taken close to the stream, in the riparian buffer zone, and in the uplands. These cores were taken to refusal to make sure all three layers of interest (surface legacy sediment, subsurface legacy sediment, and buried hydric soil) at BSR were represented in each core. After sampling, each core was divided into the three separate layers, and from the middle of each layer a ~20 cm increment of soil was collected. Each sample increment was placed in a separate, sealable plastic storage bag, and labeled. Sampling from the middle of the bottom two layers ensured that boundary effects would be avoided. Surface soils, however, were sampled from the top of each core to a 20 cm depth. The sample bags were weighed and their masses recorded. Soil samples were homogenized through the plastic bags by hand. Once homogenized, subsamples were taken by each collaborator in order to proceed with his or her designated analyses. ~100 grams of soil was retained for this project.

While in the field, 2 empty 120 mL Wheaton vials were filled with ~10-15 g of fresh soil for each sample and soil weights were recorded. One Wheaton vial was labeled as the "fresh" sample, while the other vial was labeled as the "air-dry" sample. Fresh samples were immediately capped, with the time of capping recorded. The air-dry samples were left uncapped and all samples, fresh and air-dry, were returned to the lab. KCl extractions were also carried out in the field as described below. After removing these 3 subsamples from the plastic bags containing ~100 g of sample, the bags with the remainder of soil were stored in ice-filled coolers

in the field for transportation to the laboratory. Samples were kept cool in order to slow down possible microbial processes, which could affect nutrient concentrations by changing redox conditions, and also to keep the soils from drying out, so field moisture could be measured later. When brought back to the laboratory, samples were placed in a 4°C refrigerator until further processing could take place.

In September 2010 more samples were collected at BSR from the bank-face of the stream only. These extra samples were taken because low activity levels in the bottom soils were observed in the April 2010 samples. This trend needed to be tested for soils known to be hydric soils. Sampling along the stream bank would guarantee that collected bottom soils would be hydric soils. Soils from each of the three layers of interest were collected on September 7th by inserting a hand-held core horizontally into the middle of each layer. This was repeated at 5 different positions along the bank-face of the stream. The stream bank faces at the sampling sites were cleared of debris and plant roots before collection. Coring in the middle of each layer guaranteed that each separate layer sample was composed entirely of the surface legacy sediment, subsurface legacy sediment, or buried hydric soil. Approximately 200 g of soil was collected for each sampled layer, and all was retained for analytical use. Samples were placed in plastic sample bags and properly labeled with the bank-face site number, sample depth and layer, and date. After collection, samples were immediately placed in a cooler filled with ice and transferred to a 4°C refrigerator upon return to the laboratory. The same procedures as described above involving respiration, inorganic N concentrations, and GWC were determined for the September samples. Analytical methods that measured community level physiological profiles, enzyme activities, LOI, and total C and N levels were also undertaken to further examine the bank-face soils. A number of analyzes, described in depth below, were carried out on both sets

of collected samples, those from April and September 2010, which provided useful foundational data about N cycling in hydric soils overlain by legacy sediments.

Gravimetric Water Content (GWC). Measuring the water content of the soil is fundamental to understanding basic biogeochemical processes. The thermogravimetric method, which measures the gravimetric water content (GWC), as it is commonly referred to, in terms of oven-dried samples, is the oldest established, and the only truly direct method, for measuring the water content of soil (Gardner et al., 2000). Widely accepted as referring to the water that evaporates from the soil when heated to between 100-110°C (105°C is now the standard) until reaching a constant weight, the term 'soil gravimetric water content' is based on the thermogravimetric method (Gardner et al., 2000). The temperature range was not chosen based upon scientific consideration of the drying characteristics of soil, but rather because such temperatures ensure evaporation of the free water and the oven-drying procedure is of relative ease (Gardner, 1986).

GWC was determined for all samples, those collected in April 2010 and September 2010. Fresh soils had been homogenized in plastic bags in the field, and then placed in a cooler filled with ice for transport to the laboratory. Once the samples reached the lab ~10 g of fresh soil was weighed into a pre-weighed aluminum tin. The samples were placed in a 105°C oven for ~48 hours and left to dry to a constant weight. When the samples were removed from the oven they were placed in a desiccator until they had cooled to room temperature. The now dry sample + aluminum tin were then weighed and recorded as the dry weight. To account for any rocks that may have skewed the recorded weights, the dried samples were wet sieved using a 2 mm sieve, and any rock material >2 mm was placed in a pre-weighed aluminum tin and placed back in the 105°C oven for an additional 48 hours. After all the water had evaporated from the rocks the

samples were placed in a desiccator to cool and then the rock + tin weight was recorded. To determine the GWC for each sample the dry rock weights were subtracted from the soil weight so all calculations would reflect rock-free soil weights. GWC was calculated as: GWC = g H_2O/ g oven dry soil = (fresh weight – dry weight)/ dry weight. To convert fresh soil weights (which were recorded for certain methods) to dry soil weights for calculation purposes the GWC was needed. The following conversion equation was used: dry weight = fresh weight/ (1 + GWC).

Respiration Rates Determined Through Incubations. Laboratory incubation experiments have long been used to examine net mineralization in the absence of plant uptake and under conditions that minimize gaseous and leaching losses (Stanford and Smith, 1972). Such experiments are useful for comparing the N supplying potential across soil profiles (Hart et al., 1994). Incubations are performed under artificial conditions of constant temperature and controlled moisture. In general, incubation techniques include incubating soil samples with the least possible disturbance in order to estimate the net amounts of C, NH_4^+, and NO_3^- that accumulated in each sample. Such methods involve the determination of initial soil inorganic N concentrations, which is followed by the incubation of the soil under a defined temperature and moisture regime. Respiration is monitored over the incubation period, and finally when the incubation is finished the amount of inorganic N produced during the incubation period is determined, allowing for the calculation of net rates of accumulation.

Incubation periods can vary from a week to over a year. The incubations utilized for this project were carried out over 7 days. While only a small portion of potentially mineralizable N is released during such short-term incubations, results tend to reflect the relative N-supplying capabilities of soils (Stanford and Smith, 1972). It has also been shown that 7 day incubations produce net N rates that correlate well with gross N rates (Booth et al., 2005). The relationship

between the two rates is much steeper for 7 day incubation than for 30 day incubations. It has been suggested that increases in inorganic N pools would be inhibited once a certain amount of N has been mineralized (Booth et al., 2005). With longer incubations the correlation between net N rates and gross N rates may decline.

As described above, while in the field, samples were separated into the following subsamples: 1) 10-12 g were extracted immediately extracted in 2.0 *M* KCl (to serve as the time-zero, T_0, extract); 2) 10-12 g were measured into a 125 mL Wheaton vial and then sealed with a 5 mm diameter Suba-Seal septum; and 3) 10-12 g were measured into another 125 mL Wheaton vial and left to air dry. Empty Wheaton vials were also capped in the field to serve as blanks. The sealed Wheaton vial with the "fresh" sample was taken back to the laboratory and the CO_2 released over a 7 day incubation period was monitored. Soil samples were placed in a dark cabinet at room temperature (20-22°C) for all incubations. After the final CO_2 measurement the soil was extracted with 100 mL of 2.0 *M* KCl (to serve as the final time, T_f, extract) in order to determine net nitrification by comparing the NO_3^- concentration in the T_0 extract to that in the T_f extract. NH_4^+ concentrations were also measured to determine the net rate of NH_4^+ accumulation within the soil samples. The other Wheaton vials containing the "air-dry" sample were weighed over 7 days until constant mass was reached. Once 7 days had passed the air-dry samples were rewetted to field moisture weights using deionized water, sealed with the 5 mm septa, and another 7 day incubation was conducted to monitor CO_2. A 2.0 *M* KCl extraction was also carried out at the end of the incubation. These air-dried samples were used to determine soil locations that had the potential to generate nitrate pulses after droughts.

All CO_2 measurements were taken with the use of a LI-COR 7000 CO_2/H_2O Gas Analyzer. The LI-7000 is a differential, non-dispersive infrared gas analyzer which can be

utilized to measure the difference between the CO_2 concentrations in a reference cell and a sample cell. CO_2 measurements are based on the difference in the absorption of infrared radiation passing through these two gas sampling cells (LI-COR, 2007). The analyzer has the ability to resolve very small differences in CO_2 levels, and thus it is very useful in measuring gas exchange in soils. Once Wheaton vials were sealed, the initial CO_2 concentrations were measured for each incubated sample. After ~2 days, the CO_2 concentrations in the headspace of the sealed incubated Wheaton vials were measured. The vials were uncapped for ~1 hour, then resealed and CO_2 levels measured once again. This procedure was repeated until the 7 day incubation period was over. These time-course experiments were used to determine the period over which CO_2 accumulations became linear for each set of soils. The CO_2 in each sample is linearly related to the area under the curve (the integral). This number is related to CO_2 by constructing a calibration curve, which consists of injecting known concentrations of CO_2 into the machine and plotting a regression. Initial and headspace CO_2 as outlined above were measured using a LICOR 7000 IRGA.

The above incubations and respiration measurements were repeated for the samples collected in September 2010. After examining the respiration rates from the fresh and air-dry, rewetted samples collected in April 2010, an extra component was added for analysis for the stream bank face samples. The third scenario that was tested only for the bank-face samples involved air-drying soil in Wheaton vials, and then instead of rewetting and incubating the samples, the samples were extracted immediately after drying with 2.0 *M* KCl. These samples thus provided new data about air-dried, not rewetted samples. This extra experiment was useful in determining at which stage different N forms were released; i.e. was it the drying or rewetting component that could lead to spikes in N release.

Potassium Chloride (KCl) Extraction Method. The determination of exchangeable NH_4^+ and NO_3^- involves the extraction of these inorganic forms of N followed by analysis of the extract by spectrometric procedures. Exchangeable NH_4^+ and NO_3^- in soils have been defined as the NH_4^+ and NO_3^- extractable by 2.0 *M* KCl at room temperature (Bremner and Keeney, 1966). The use of 2.0 *M* KCl has many practical advantages. Only one extraction of a soil sample is needed to analyze both NH_4^+ and NO_3^- when KCl is used as the extractant. The method is also widely used because it can easily extract inorganic N from the soil in little time, and extracts can be stored for long periods of time before analysis (Dorich and Nelson, 1984). This KCl extraction method is based on the principle that to measure exchangeable ions the exchange complex must be saturated with ions which force the exchangeable ions already present on the charged surfaces into solution (Hendershot and Lalande, 1993). Specifically this extraction adds a salt solution (2.0 *M* KCl) to the soil, displacing ions of interest (NH_4^+) from their cation and anion exchange sites. Most NO_3^- in soils with low anion exchange capacities is water soluble. For this reason water or salt solutions, like 2.0 M KCl can be used to extract NO_3^- from the soil (Griffin, et al. 1995). Each extraction solution has advantages and disadvantages.

The KCl extraction method used is based on that outlined by Bremner and Keeney, 1966. The 2.0 M KCl solution for this project was prepared in one large carboy that held 20 L of solution. The molecular mass of KCl is approximately 74.55 g/mol. The mass of reagent grade KCl that had to be dissolved to make a 20 L batch of 2.0 *M* KCl was calculated as follows: $\frac{74.55g}{mol} \times \frac{2.0mol}{L} \times 20L = 2982g$. Thus 2982 g of KCl was dissolved in deionized water (DI) and diluted to 20 L with more DI water. Deionized water is water that has had its mineral ions (such as calcium, sodium, chloride, etc.) removed. Ions in water have the potential to cause interferences, as they can switch places with other ions that may be of interest. By using

deionized water such interferences are eliminated, allowing more accurate concentrations to be measured.

Soil samples were to be placed in the 2.0 *M* KCl extraction solution as soon as possible after being collected. To ensure the rapidity of this process all individual KCl solution aliquots were measured ahead of time and refrigerated until the time of sampling. To create a 1:10 soil: extractant ratio, it was planned that ~12-15 g of fresh weight soil (~10 g of dry weight soil) would be added in the field to the bottles with the pre-portioned KCl aliquots. About 1 week prior to sample collection, 100 mL of 2.0 *M* KCl was measured into a 250 mL polypropylene wide-mouth bottle, capped, and placed in the refrigerator. During sample collection the bottles of KCl were placed in a cooler which contained ice in order to keep the solutions at colder temperatures. Soil samples were collected, placed in plastic Ziploc bags, and homogenized by hand through the bag. To collect representative samples, soil was scooped from several places within each sample bag. Between 12-15 g of soil was added to the bottled KCl solution. Soil moisture and rock content were not known at the time of sampling, so 12-15 g of fresh soil was used for extraction to guarantee that each fresh sample was at least equivalent to 10 g of oven dry <2 mm. Some bottles containing KCl solution were left empty of soil for use as blank checks. Samples were refrigerated until the next steps in the method could be performed.

The samples were removed from the refrigerator and placed on a reciprocating horizontal mechanical shaker for an hour. NO_3^- can be readily extracted from soil after only a short period (5-10 minutes) of shaking with a KCl solution (Bremner and Keeney, 1966). Exchangeable NH_4^+ acts as the limiting factor. According to trials conducted by Bremner and Keeney (1966) concentrations of NH_4^+ become stable after about 60 minutes of shaking, and do not increase even if shaking time is increased past 1 hr. The standard protocol for simultaneously extracting

NH_4^+ and NO_3^- has utilized this information, and a shaking time of 1 hr. is the accepted interval needed to completely extract the inorganic N forms.

After shaking the samples were let to sit until the sediment had settled to the bottom of the wide-mouth bottles. Whatman Grade 1 qualitative filter paper circles with diameters of 125 mm were used for filtration. Such filter paper is composed of cellulose fibers, which allow for the use of gravimetric analytical techniques to determine and identify materials. Grade 1 filter paper is widely used for routine applications, like separating solid soil particles from associated extracting liquid, and is characterized by medium retention and flow rate. Each filter paper was folded into a conical shape, placed into a plastic funnel, and set on a filter stand. In this position the filter paper was pre-leached with the extractant matrix, 2.0 M KCl, to rinse away any possible contaminants from the filter paper prior to sample filtration, and to help the filter paper better adhere to the funnel. The settled samples were poured into the filtering funnel units in such a way to minimize the movement of sediment into the filters; pouring only supernatant and small particles in solution into filters was the goal. Underneath each filtration unit was a 120 mL polypropylene specimen cup used to catch and store the filtrate. For the samples collected in April 2010, ~50 mL filtrate was retained for sample analysis. The other ~50 mL portion of the sample was poured into a labeled 125 mL VWR wide-mouth High Density Polyethylene (HDPE) bottle into which 3 drops of H_2SO_4 were added. These samples were then sent to the Ground Water and Ecosystem Restoration Division (GWERD) of the U.S. Environmental Protection Agency (EPA) in Ada, Oklahoma for denitrification analysis by a collaborator, Dr. Kenneth J. Forshay. All of the ~100 mL of solution filtered from the stream bank samples collected in September 2010 were retained for analysis.

Rock weight in each extraction sample had to be taken into account for calculation purposes. For this reason, at the end of the filtration process each filter paper's soil residue was examined to make sure there were no rock fragments present. If there were any rocks they were removed and placed back into the appropriate wide-mouth bottle with the remaining sample solids. The fraction of residue on the filter paper that was not rock was not needed, and thus the filter paper was discarded. The residual soil in each sample bottle was wet sieved through a 2 mm mesh sieve. The greater than 2 mm fraction that remained on the top of the sieve was considered non-soil; it was categorized as rock. This fraction size was placed in a pre-weighed aluminum tin, and the total weight of the rock + tin was recorded. The tins were placed in a 105°C oven to dry for 48 hours in order for the sample to reach and maintain constant mass. Once removed from the drying oven the tins were placed in a desiccator to cool. Once cool the samples were removed from the desiccator and re-weighed. Rock weight was determined as: (Final Weight of Rock + Tin) – (Tin Weight). The extracted solution was placed in the freezer until ready for NH_4^+ and NO_3^- analysis, at which time samples were set out to warm them to room temperature for accurate pipetting.

Inorganic Nitrogen Concentrations. The concentrations of NH_4^+ and NO_3^- (the inorganic forms of N) in 2.0 *M* KCl extracts were measured by employing two spectrometric procedures – the salicylate microplate technique and the vanadium (III) chloride microplate photometer method. Both procedures were carried out on a Thermo Scientific Multiskan EX microplate photometer using 96-well microtiter plates that handled volumes up to 300 μL, and all chemicals were of A.C.S. reagent grade. The salicylate procedure had been in place in the Kaye lab for over a year. The vanadium (III) chloride method was developed in winter 2009-2010 with correspondence from Timothy Doane (University of California, Davis). With the use

of microtiter plates both approaches were carried out using a batch process, which allowed large numbers of samples to be analyzed, while also eliminating the potential for carryover contamination between analyzes (Sims et al., 1995). Thus, the use of two separate methods to measure NH_4^+ and NO_3^- concentrations proved to be efficient and unproblematic.

Calibration Standard Solutions. Solutions with known concentrations of NH_4^+ and NO_3^- had to be made prior to carrying out the salicylate and vanadium (III) chloride methods. Individual sets of standards had to be prepared for both NH_4^+ and NO_3^- as the two N forms could not be combined in one solution. Since NH_4^+ and NO_3^- were run separately, standards were made according to the expected concentrations of each respective ion. All standards were made in a 2.0 *M* KCl matrix as that was the same matrix used to extract exchangeable ions from the collected samples. Stock solutions of 1000 ppm NH_4Cl and 1000 ppm KNO_3 made in deionized water were used to make a more diluted solution from which the calibration standards were made. The amount of stock solution that needed to be transferred was so small that all the calibration standards could be diluted in the same matrix that was used for sample extraction.

Standards were made through a series of dilutions using the general dilution equation, $C_1V_1 = C_2V_2$, in which C stands for concentration and V stands for volume. In order to avoid pipetting small aliquots of solution, which increase the likelihood of pipetting errors, the 1000 ppm stock solutions were first diluted to 20 ppm. The rest of the standards were made using the 20 ppm solution as the "stock" solution. Using the above dilution equation 100 mL of 20 ppm solution (NH_4^+ or NO_3^-) could be made from the stock solution of 1000 ppm (NH_4Cl or KNO_3) by pipetting 2 mL of the 1000 ppm solution into a 100 mL volumetric flask [(1000ppm)*(V_1) = (20 ppm)*(100 mL), so V_1 = 2 mL] and making to volume with the matrix solution, 2.0 *M* KCl. From the more diluted 20 ppm NH_4Cl "stock" solution the standards 0, 0.1, 0.2, 0.5, 1.0, and 5.0

ppm were made for calibrating the microplate photometer for NH_4^+ analysis. Based on available data it was found that NH_4^+ concentrations for soils in the Lancaster area tended to be below 5 ppm. Thus, the calibration standards were designed to frame the range of 0-5.0 ppm based on the concentrations expected for the samples, while also falling within the range of detection for the microplate equipment. For NO_3^- the calibration curve was constructed by making dilutions from the 20 ppm KNO_3 "stock" solution to make standards that were 0, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 ppm. (The 0 ppm calibration standards for both NH_4^+ and NO_3^- were N-free, as they were composed of only the 2.0 *M* KCl matrix solution.) The calibration curve for NO_3^- becomes non-linear above 2.0 ppm, and thus if any samples had NO_3^- concentrations greater than 2.0 ppm they were diluted and rerun. An alternative to diluting the samples could have consisted of making a new set of standards that were all above 2.0 ppm. At this higher concentration range the calibration curve once again exhibits a linear relationship between absorbance and concentration. The samples that had originally been measured as having concentrations >2.0 ppm could be analyzed again using the second, higher calibration. However, there were few samples that had concentrations above 2.0 ppm, and for this reason diluting the samples was a more practical option than making new standards that would produce a calibration curve capable of measuring concentrations >2.0 ppm.

Salicylate Microplate Method. There is no specific dye to directly measure NO_3^- via colorimetric techniques, so most methods rely on the reduction of NO_3^- to another N form. The salicylate microplate method measures NH_4^+, and when combined with Devarda's alloy can be used to completely reduce NO_3^- to NH_4^+ followed by a colorimetric determination of NH_4^+ (Sims et al., 1995). This one method can thus be used to measure NH_4^+ directly, and NO_3^- by difference. While Devarda's alloy presents no disposal problem as it is composed entirely of

non-regulated metals (Cu, Zn, and Al), pipetting challenges can arise while dispensing the powder, or transferring the sample solution to remove the Devarda's alloy for analysis. While the addition of Devarda's alloy can be utilized to measure NH_4^+ directly and NO_3^- indirectly, only NH_4^+ concentrations were determined using this salicylate procedure in order to eliminate the use of Devarda's alloy. The salicylate method involves a series of reactions that form 5-aminosalicylate (MDHMH, 2010). Sodium nitroprusside acts as a catalyst for the reaction of NH_4^+ with salicylate and hypochlorite, and sodium citrate is used to prevent metal hydroxide precipitation (Bower and Holm-Hansen, 1980). This forms a blue-green colored dye when oxidized that is proportional to the amount of NH_4^+ present in the sample. NH_4^+ standards in 2.0 *M* KCl were prepared in order to produce a standard calibration curve on the microplate reader. Calibration standards and unknown samples were added in predetermined amounts based on calibration standard dilution concentrations. For this project 120 μL aliquots of sample were added to a 96-well plate.

Several reagents had to be made in order to carry out the reactions necessary to measure NH_4^+ using the microplate photometer – 2.0 *M* sodium hydroxide, hypochlorite, citrate, and salicylate-nitroprusside. Needed for the hypochlorite reagent, 2.0 *M* sodium hydroxide was made by dissolving 8 g of sodium hydroxide pellets in deionized water and made to volume in a 100 mL volumetric flask. The hypochlorite reagent had to be made fresh each day of use. It was made by combining 1 g sodium tribasic phosphate dodecahydrate, 2 mL of 2.0 *M* sodium hydroxide, and 10 mL of bleach in a 100 mL volumetric flask and bringing it to volume with deionized water. The salicylate-nitroprusside was made by dissolving 7.813 g sodium salicylate and 0.125 g sodium nitroprusside in a 100 mL volumetric flask that was made to volume with deionized water. If placed in a light-proof container and refrigerated, the salicylate-nitroprusside

reagent could be stored for up to 4 weeks. The last reagent that needed to be made was citrate. Similarly to the salicylate-nitroprusside reagent, the citrate reagent could be stored in the refrigerator in a light-proof container, but would only be usable for ~2 weeks. In a 100 mL volumetric flask 5 g trisodium citrate dehydrate and 2 g sodium hydroxide pellets were dissolved in deionized water and brought up to volume to produce the citrate reagent.

After adding 120 μL of sample to each well, 25 μL of citrate reagent, 50 μL of salicylate-nitroprusside reagent, and 25 μL of hypochlorite reagent were added, too. The amount of blank matrix, in this case 2.0 *M* KCl, depended on the amount of sample initially placed in each well, and the total of sample plus blank matrix had to total 175 μL. Thus, 55 μL of 2.0 *M* KCl was added to each well containing an unknown sample or calibration standard. Check standards with known concentrations of 0.5 ppm NH_4^+ were pipetted into random empty wells and filled with the appropriate reagents. The check standards were included in each batch of analyses to serve as quality control measures for NH_4^+ concentrations. After all the samples and reagents had been combined, the plate then sat for about 30 minutes prior to analysis on the microplate photometer in order to allow time for complete color change. Absorbance was read by the microplate at 650 nm. Using the produced standard calibration curve NH_4^+ concentrations were then calculated for each sample by the microplate instrument.

Vanadium (III) Chloride Microplate Method. NO_3^- concentrations were determined by using the vanadium (III) chloride method (VCl_3). This method utilizes the reaction of vanadium in a 1M hydrochloric acid solution to reduce NO_3^- to NO_2^-, and as it is formed NO_2^- is captured by Griess reagents, producing a color change (Doane and Horwath, 2003). Though only capable of measuring NO_3^- concentrations, the vanadium (III) chloride method involved less procedural, and equipment considerations, as well as simpler preparation and analysis than the Devarda's

alloy method (Doane and Horwath, 2003). The vanadium (III) chloride reagent solution was prepared by pouring 200 mL of 0.5 *M* HCl into a beaker. Placing this solution on a balance, approximately 0.5 g vanadium (III) chloride was added. Directly adding the vanadium (III) chloride to the HCl solution avoided any sticking of the powder to spatulas and weigh dishes. The solution was then stirred gently until most of the vanadium (III) chloride dissolved. Placing the solution back on the balance ~ 0.2 g sulfanilamide and ~0.01 g N-(1-naphthyl)ethylenediamine dihydrochloride were added and dissolved. The produced vanadium (III) chloride reagent remains stable for ~1-2 weeks if stored in a container and placed in the refrigerator.

The transfer amount of calibration standards and unknown samples was predetermined based on the concentration used for the calibration standards. Once determined, the calibration standards and unknown samples were added to a clean 96-well plate. Blank matrix of 2.0 *M* KCl was also added to each well when necessary. The amount of blank matrix depended on the amount of sample initially placed in each well, and the total of sample plus blank matrix was always 50 μL. For this analysis no matrix solution was added as 50 μL of standards for calibration and unknown samples were pipetted into a 96-well plate. Check standards with known concentrations of 1.0 ppm NO_3^- were put in random wells to ensure that there were no measurement errors. The vanadium (III) chloride reagent was then added to each standard and sample in 250 μL aliquots. The plate was covered with parafilm and allowed to sit in a fume hood at room temperature overnight. Absorbance was read by the microplate photometer at 540 nm. The linear curve produced from the standards calibration was used to relate concentration to peak area of height (which represented absorbance), and thus calculate the NO_3^- concentration for each sample.

II. EPA Analyses

Collaborations with other institutes conducting research at BSR were established, which provided advantages to all those involved. These collaborations allowed for a larger suite of analyses to be carried out. Samples processed by other laboratories provided extra data that was utilized in interpreting the patterns found at BSR. Scientists at the U.S. Environmental Protection Agency (EPA) in Ada, OK, collaborators on the project, analyzed samples for total C and N concentrations, organic matter content, and denitrification potentials. Combining the information generated by each of these procedures not only allowed for a more comprehensive understanding of the tested soil samples, but it also served as a model from which local and regional trends in biogeochemical cycling could be explored.

Total Carbon and Nitrogen Concentrations. The Standard Operating Procedure (SOP) for determining total C and N concentrations of soil samples by the EPA is very similar to that carried out in the Kaye Lab. Soils were frozen prior to this procedure, so frozen samples were thawed in a 102-105° drying oven for at least 24 hours before analysis, and dried to a constant weight. Once dry, the samples were homogenized using a mortar and pestle. The samples were stored in a desiccator until preparation and analysis were undertaken. Aluminum capsules were precombusted at 550°C in a muffle furnace for 1.5 hours, for each sample being analyzed. 10 mg of homogenized sample was weighed to the nearest 0.001 mg into a precombusted aluminum capsule using an ultra-micro balance. The top of the aluminum capsule was crimped with Teflon-coated flat-tipped forceps, and placed into 96-well microtiter plate. Samples were shipped to U.C. Davis for total C and N analysis as well as isotope analysis.

Organic Matter Content. Organic matter content was determined using the samples that were incubated for denitrification analysis. Jars contained ~75 mL of soil, and 500 mL of

deionized water was added to each jar following incubation in order to measure the headspace volume. Following this, the contents of the full jar were poured into a large pre-weighed tin. All the remaining residue in the jar was rinsed into the tin with a squirt bottle containing deionized water. The tin containing the water and soil was then placed in a drying oven at 50°C and left to dry for about a week. Once dry, soil samples were taken from the large tin, and weighed into smaller tins. Samples were placed back into the oven until they reached constant weight. The samples were then removed from the drying oven and placed in a desiccator to cool to room temperature. The dry soil + tin were weighed, and the mass recorded as dry tin weight. The samples were then placed in a 500°C muffle furnace for 4 hours, removed, and placed into the drying oven overnight. The new weight + tin were weighed the next day, and labeled as new tin weight. The organic matter content of the soil was calculated as the difference between dry tin weight and new tin weight.

III. Analyses of September 2010 Samples

Total Carbon and Nitrogen Concentrations. The total concentration of C and N in each solid sample collected in September 2010 was determined by dry combustion elemental analysis. The instrument utilized for this project was a CHNS-O Analyzer – an elemental analyzer dedicated to the simultaneous determination of the percentage amount of carbon, hydrogen, nitrogen, sulfur, and oxygen. The analyzer uses a combustion method to convert the sample elements to simple gases (CO_2, H_2O, N_2, and SO_2), which involves the complete and instantaneous oxidation of the sample by "flash combustion" (ICES, 2008). To prepare soil samples for the CHNS-O Analyzer, soil subsets were dried (105°C to constant mass), dry sieved (< 2mm), and ground to the consistency of talc using a roller-mill grinder. Soil samples were placed along with two metal roller pins, one wide and one thin, into 20 mL scintillation vials.

All the vials were placed in a larger container and set on the roller-mill on a medium setting for 1 week. This grinding procedure ensured that the samples had been ground to a fine powder (<150 μm). Sample variability is usually a concern for total C and N analysis due to the small sample size required for the combustion analyzer, and thus the grinding step served to homogenize the samples so they would each have a uniform structure and composition throughout their entire sample matrix.

Once ground, the soil samples were weighed into 5 x 8 mm tin (Sn) capsules on an analytical balance. Soil weights are dependent upon whether the sample is more organic or more mineral based. Samples of a higher weight generally have combustion problems due to their size. Thus, about 10-12 mg of ground sample was weighed so possible combustion setbacks could be avoided, as well as so C and N concentrations would fall in a range suitable for measurement on the CHNS-O Analyzer. Sample carousels used to drop the samples into the CHNS-O Analyzer utilize pneumatic processes. These carousels only hold samples of a certain size so it was necessary to carefully prepare each sample tin. After a soil sample was weighed into a tin capsule it was handled with forceps which were used to clasp the top of the tin closed. The forceps were also used to shape the tin sample into a ball so that it would not get caught as the sample was being injected into the combustion column of the elemental analyzer.

After the tin capsules were prepared, the C and N concentration (in mg C/ kg oven dry soil) of the sieved, ground soil was then determined by dry combustion elemental analysis. The tins capsules were loaded into the auto-sampling carousel and mounted on the CHNS-O Analyzer. Once the machine warmed up, the capsules were individually dropped into a quartz tube at ~1020°C with constant helium flow (the carrier gas). To guarantee almost complete combustion of samples, a strong oxidizing environment had to be achieved. A few seconds

before the sample drops into the combustion tube, the air stream is enriched with a measured amount of high purity oxygen which produces the necessary environment for combustion. The combustion gas mixture is then driven through subsequent oxidizing and reducing zones, and the resulting form is detected by a Thermal Conductivity detector (ICES, 2008). Instrument standards were used to calibrate the CHNS-O Analyzer for soil samples. Check standards (~0.9 mg) and references (~10 mg) with known C and N concentrations were also used to ensure there was no measurement drift of the instrument.

Loss on Ignition (LOI). Sequential loss on ignition (LOI) is a common and widely used method to estimate the organic and carbonate content of soils (Heiri et al., 2001; Dean, 1974). LOI corrects for loss of volatile compounds, like water and organic matter. The LOI protocol provides a fast and inexpensive means of determining organic and carbonate contents with precision and accuracy comparable to other, more expensive and sophisticated methods (Dean, 1974). LOI measurements are based on the sequential heating of soil samples in a muffle furnace, from which the weight percent of organic matter and carbonate materials can be determined. For this project, however, only the first step in sequential LOI was carried out in order to estimate the organic matter content of the soil samples. This method is based on differential thermal analysis, which has shown that organic matter begins to ignite around 200°C and is completely depleted once temperatures reach over 450°C (Santisteban et al., 2004). The organic matter LOI was reported as the percent of organic matter, which was assumed to be the same as the total percent of loss on ignition calculated from only the first step in the LOI sequence.

To measure organic matter content for each sample from the bank face of BSR using LOI a portion of the fine powder soil fraction ground on the roller mill was used. Approximately

1.0000 – 3.0000 g of each ground < 0.4 mm sample was accurately and precisely weighed to the ten-thousandths place on an analytical microbalance into a clean and labeled aluminum tin that was previously precisely weighed. These samples were then placed in a 105°C oven for about 24 hours. Once the samples reached constant mass they were placed in a desiccator to cool down. Placing the heated samples into a desiccator ensured that no moisture would reenter the soil, and thus would not increase the dry weight measurements. After the samples were completely cooled the aluminum tins containing the soil were reweighed and labeled as the (105°C + tin) weight. Samples were then transferred to a cold muffle furnace that was set to a temperature of 450°C. At such high temperatures organic matter is oxidized and combusted into carbon dioxide and ash (Heiri et al., 2001). Once the temperature was reached the samples were left in the furnace for 16 hours. The samples were removed from the furnace and once again placed in a desiccator to cool. When the samples were completely cooled the weight of the soil and tin were recorded and labeled as the (450°C + tin) weight. Tin weights were subtracted from all samples, and the weight differential between the two temperatures was then calculated as: LOI% = ((105°C weight) – (450°C weight)/(105°C weight) * 100, and reported as percent organic matter.

Community Level Physiological Profile (CLPP). Soil microbial community diversity has been used to assess the 'health' or 'quality' of soils (Chapman et al., 2007). Recognized as the essential living component of the soil, the size and activity of microbial communities can be utilized as indicators of soil conditions. In particular, the assessment of the functional diversity of the soil is seen as key to identifying the quality of the soil (White and MacNaughton, 1997). Functional diversity can be directly related to the activity of the soil microflora, and provides information concerning the functioning of the microbes involved in cycling carbon. Community

level physiological profiles (CLPPs) are used to assess soil functional diversity. CLPPs of soils are determined by examining the utilization of different carbon substrates by measuring the amount of CO_2 that is respired by the soil microorganisms that are decomposing the different introduced substrates (Campbell et al., 2003). The soil microbial community is compromised of many different microorganisms that have diverse abilities to decompose certain substrates. By adding different substrates to soil samples it is possible to assess the catabolic diversity of the community and obtain a fingerprint of the soil, or a CLPP (Degens and Harris, 1997). Thus, the activities measured within the soil reveal the ability of different soil microbes to metabolize various sets of simple and/or complex organic compounds. This project utilized the MicroResp™ technique to determine CLPPs, as it offered a convenient, rapid, and sensitive method for measuring responses of whole soil communities (Tlili et al., 2011).

MicroResp™ has an advantage over the Biolog system, a method long used for CLPPs, in that substrates are added directly to soils in the MicroResp™ procedure, as opposed to having to utilize soil slurries. While the Biolog method has the ability to test the utilization of 95 different substrates simultaneously its dependence on inefficient extraction, the physiological status of inoculated cells, the subsequent growth of cells, and the relevance and concentrations of the C substrates used have long be questioned (Campbell et al., 2003). The reliance on soil suspension extractions and the consequential growth of organisms biases the results of the Biolog approach toward organisms that are readily extractable and able to develop rapidly within the aqueous conditions utilized (Campbell et al., 2003). The MicroResp™ method, however, makes use of whole soils which allows for more immediate responses to substrates to be obtained without the need for extraction or culturing of organisms. Activity, rather than growth,

is measured using the MicroResp^{TM} method, which can be used to better assess microbial community structure and function.

A CLPP of each soil layer of only the bank-face samples collected in September 2010 was conducted to better understand the diversity of microbial substrate use across the soil profile at BSR. To analysis the functional diversity of the bank face soils of BSR 15 substrates were separately added to each soil sample. The complexity of substrates ranged from very labile molecules like glucose to more recalcitrant molecules, such as lignin. The CLPP of the BSR site was obtained by utilizing the following substrates, which were all C sources: D-glucose, citric acid, ascorbic acid, urea, asparagines, L-cysteine, glycine, lignin, pepsin, N-acetyl glucosamine, α-ketobutyric acid, malic acid, oxalic acid, tannin, and humic acid. The activity levels produced in the presence of these substrates were compared to the reaction of a 16th blank substrate, which consisted of deionized water. The soils with substrates were all incubated under the same conditions. This allows one to measure the ability of each soil microbial community to utilize the above suite of substrates.

For measuring microbial activity it was recommended that the moisture content of the soil samples should within the range of 30-60% of the maximum water holding capacity (WHC). To ensure samples were not too wet, nor too dry, for use in the MicroResp^{TM} method, the WHC of each sample was determined. For each sample, a mass of ~10-15 g fresh soil sample was weighed and spread evenly across the surface of a Buchner funnel containing a 125 mm diameter Whatman Grade 1 qualitative circular filter paper. The Buchner funnel was then placed on top of an Erlenmeyer filter flask. The soil sample was saturated with deionized water, and then the flask was attached to an electric vacuum set at 33 kPa to mimic WHC. The water was drawn into the Erlenmeyer filter flask, and the vacuum turned off and removed once water flow

dissipated. The soil, having reached WHC at 33 kPa, was then reweighed. Prior to beginning the CLPP procedure each soil sample was wetted with deionized water to 60% WHC. The soils samples were then scooped into the deepwell plates utilized for the MicroResp^{TM} method, and weighed. Each of the deepwell plates filled with soil samples were covered with Parafilm and incubated at 25°C in a dark cabinet for ~5 days in a large sealed box containing a beaker of self-indicating lime, and lined with wet paper towels. After the incubation was complete, the soils were ready to be analyzed for CLPP.

While samples were being incubated detection plates were prepared. An indicator solution was made by dissolving 18.75 mg cresol red, 16.77 g potassium chloride, and 0.315 g sodium bicarbonate in 900 mL of deionized water over low heat (<65°C), and then diluting the solution to volume in a 1000 mL volumetric flask. A 3% Purified Agar solution was also prepared by dissolving 3 g of agar in 100 mL of deionized water. The agar was dissolved by setting the mixture in an autoclave set at 121°C for 20 minutes. The agar was then allowed to cool to 60°C in a water bath. A solution consisting of a 1:2 ratio of agar: indicator was needed, so 200 mL of the indicator solution was poured into a large beaker and warmed to 60°C in a water bath. Once the temperature for both the agar and indicator solutions had equilibrated they were transferred into a beaker and mixed thoroughly. The heat of the mixed solution was maintained at 60°C and a stir bar added to the solution to ensure the solution was constantly being stirred. An automated 8-channel pipette was used to dispense 150 µL (using three 50 µL aliquots) of the mixed solution into each column of a microtiter plate. Once a whole plate was filled with the detecting solution, the plate was stored in the dark at room temperature inside a desiccator containing self-indicating soda lime and a beaker of water. The plate was left uncovered in the desiccator and allowed to equilibrate for 2-3 days before use. To make sure the

plate was usable it was analyzed on a microplate spectrophotometer at a filter of 570 nm. Plates were discarded if the coefficient of variation (CV) was >5%, indicating that there was a non-uniform amount of agar in the wells. Detection plates could be utilized more than once as long as they were left to "recharge" for ~12 hours and their CV was checked again before reuse.

Carbon substrates were also prepared during the time in which soil samples were incubating. The substrates were prepared as 30 mg per gram of soil water, which differed for each substrate. The concentration of carbon source needed for each sample was calculated as the weight of H_2O per g of soil in each well multiplied by the required 30 mg per gram of soil water for each substrate, resulting in a mg unit. Though all substrates were added directly to the soil samples in 25 µL aliquots, it was easier for dissolution purposes to make larger batches of substrates (Macaulay Scientific, 2010). For this reason, the required amount of substrate for each soil, which was calculated using the above equation, was converted to a g unit and the substrate was then dissolved in 25 mL of deionized water. Once all substrates were prepared (D-glucose, citric acid, ascorbic acid, urea, asparagines, L-cysteine, glycine, lignin, pepsin, N-acetyl glucosamine, α-ketobutyric acid, malic acid, oxalic acid, tannin, and humic acid) they were stored in a 4°C refrigerator until ready to be used, at which time they were removed and allowed to warm to room temperature.

After detection plates were equilibrated and soil samples had finished incubating, the analytical steps of the MicroResp^{TM} method were undertaken. 25 µL of each substrate was dispensed into the corresponding wells of the deepwell plate. Samples were run in triplicate to ensure the best estimate of the mean absorbance for each carbon source. Once all substrates were added to the soil samples, a MicroResp^{TM} seal was applied to the deepwell plates to allow gas transfer between the samples and the detection plate. The seal is a PTFE coated rubber mat

that was constructed in such a way that each well of the deepwell plate corresponds with one well on the detection plate, allowing the two plates to hermetically seal together. Before attaching the detection plate onto the opposite side of the MicroResp^{TM} seal, its absorbance was read at a 570 nm wavelength on a microplate spectrophotometer. The resultant data from the detection plate was labeled as the absorbance at time zero (At0). The above steps were repeated for all the deepwell plates that contained soil samples. Even pressure was applied to each {detection plate – MicroResp^{TM} seal – deepwell plate} unit by placing heavy objects, like textbooks, on top of them. This ensured that each unit was sealed correctly. All MicroResp^{TM} units were then incubated in a dark cabinet at 25°C for 6 hour stretches over a period of 24 hours. After each 6 hour incubation period the detection plates were removed and peeled off of the MicroResp^{TM} seal. The detection plates were once again read on the microplate spectrophotometer and the results saved as At6. The detection plate, containing the gel-based bicarbonate buffer with the indicator dye, responded to the pH change within the gel, resulting from carbon dioxide that evolved from the soil (Chapman et al., 2007). Thus the color change was measured and reported on a microplate reader as a different absorbance. New detection plates were read for each of the remaining incubation cycles and labeled as $At_0$6, $At_0$12, and $At_0$18 depending on the incubation schedule. The plates were attached to the MicroResp^{TM} seals again, and the units were then incubated for periods of 6 hours. After each incubation round the detection plates were once again read and labeled as At12, At18, and At24, in accordance with the succession of time.

All CLPP results are reported as CO_2 production rates. All the data was normalized (Ai) for the initial and final times for each separate 6 hour incubation period. This was achieved by dividing the final absorbance data by the initial absorbance data and multiplying by the mean of

the absorbance reading at the initial time. The following formula was then used to convert the normalized data to $\%CO_2$: $\%CO_2 = A + B / (1 + D \times Ai)$, where A = -0.2265, B = -1.606, and D = -6.771 (Macaulay Scientific, 2010). These parameters were obtained from a calibration measured over 6 hours at 570 nm, using soils with pH <7 and the same detection solution as described above. The formula and parameters were determined for a linear-to-linear standard curve fit reported by Macaulay Scientific (2010). To then convert the calculated $\%CO_2$ data to a production rate, a number of factors had to be considered: gas constants, incubation temperatures, headspace volumes in each well, fresh weights of each soil per well, incubation times, and the percent dry weight of each soil sample. Thus, the CO_2 rate (µg CO_2-C/g/h) for each soil sample was calculated as: $\frac{(\%CO_2/100)\times(vol)\times(44/22.4)\times(12/44)\times(273/(273+T)}{soil\ \ fwt\times(soil\ \ \%\ \ dwt/100)}$.

$$incubation\ \ \ time$$

Extracellular Enzymatic Activities. Extracellular enzymes are produced by microbes in order to breakdown large polymeric organic material into smaller monomers. They enable soil microbes to degrade complex substrates into low molecular weight compounds that can then be assimilated for growth (Allison, 2006). Extracellular enzymes are of particular interest from an ecological perspective because they are responsible for catalyzing the rate limiting steps of decomposition and nutrient cycling; they degrade complex molecules which can yield assimilable macronutrients (Sinsabaugh, 1994). Excreted by the cell, extracellular enzymes exist in soil solution in their free forms. Measuring the activity of these enzymes is important because they play a central role in the microbial cycling of nutrients (Arnosti, 2011). Since enzymes are the main mediators of soil biological processes, the study of their diversity and activities provides an effective approach for examining functional diversity and heterogeneity in soils.

The kinetics of decomposition in soils is largely motivated by intrinsic characteristics of plant residues (Bertrand et al., 2006; 2009). Such residues correspond to the substrates used for microbial growth and any enzymatic activities. Extra-cellular enzymes involved in the degradation, transformation, and mineralization of plant residues act to mediate the processes of microbial growth and soil decomposition (Sinsabaugh et al., 2009). Constraints on enzyme production and activity may regulate carbon degradation and the release of nutrients from complex compounds (Allison, 2006). Thus, the relationships between soil microbial activity and new or recycled carbon entering the soil can be further understood by undertaking exoenzyme assays because they provide functional information concerning the microbes present in the soil (Sinsabaugh et al., 1991).

By conducting both a community level physiological profile analysis (CLPP) and an enzymatic activity analysis, which relates microbial activity with the community's functional diversity (Dalmonech et al., 2010) two independent measures of carbon-substrate utilization could be attained and compared. For better comparison of C-substrate utilization between the CLPP and enzyme analysis, some of the extracellular enzymes utilized to determine enzyme activity were chosen because they had already been used in the acquisition of some MicroResp™ substrates. The 7 extracellular enzymes used to assess in situ microbial activity were also chosen as they represent commonly employed enzymes utilized in labile and recalcitrant C and N acquisition. The C-acquiring enzymes could be categorized as either hydrolytic enzymes, involved in the degradation of more labile substances, or as oxidative enzymes, which relate more to recalcitrant compounds (Dalmonech et al., 2010). The hydrolytic enzymes included: cellobiohydrolase (CBH) and β-glucosidase (BG), which are key C-enzymes involved in cellulose, hemicellulose, and starch degradation; *N*-acetyl-β-glucosaminidase (NAG), which is

involved in N cycling through the release of amino sugars from chitin; leucine aminopeptidase (LA), which releases amino acids from polypeptides, impacting the N cycle as well; and acid phosphatase (AP), which is a crucial enzyme involved in transformations of organic P (Dalmonech et al., 2010). The oxidative enzymes, which are involved in the depolymerization and polymerization of recalcitrant C-substances as lignin and lignin-like compounds, (Dalmonech et al., 2010) were polyphenol oxidase (PPO) and peroxidase (PerO).

The specific individual activities of each of the enzymes used to measure activity levels are much more defined than described above. Enzyme activities are interdependent, and thus, the process each enzyme undertakes greatly affects the activity levels of other enzymes in the same soil. For example, while both CHB and BG degrade cellulose, CBH depolymerizes cellulose into cellobiose by releasing disaccharides from cellulose while BG hydrolyzes that cellobiose to form glucose (Allison and Vitousek, 2004; Keeler et al., 2009). By breaking down chitin the NAG enzyme plays an important role in organic N acquisition. Similarly, AP hydrolyzes bound organic P, like PO_4^{3-}, by hydrolyzing phosphate ester bonds, which is useful in determining the magnitude to which microbes invest in the acquisition of P (Allison and Vitousek, 2004).

Enzyme Activity Assays. Extracellular enzyme assays were carried out only on samples collected in September 2010. The enzyme assays were undertaken in order to better understand the role each of the following plays in BSR bank-face soil: phosphatase, cellobiohydrolase, β-glucosidase, β-N-acetylglucosaminidase, leucine aminopeptidase, polyphenol oxidase, and peroxidase. Extracellular enzyme activities were measured with assay techniques modified from Sinsabaugh *et al.* (1991) and Allison and Vitousek (2004). The enzymes were assayed using the necessary substrates, and then measured for absorbance on the microplate spectrophotometer.

Absorbances were then used to calculate enzyme activities in terms of µmol of substrate per hour per gram of soil organic matter (µmol substrate hr^{-1} g SOM^{-1}).

Once the collected bank face soil samples were transported from the field to the laboratory they were each mixed by hand in their respective sample bags. The samples were then sieved through a 2 mm sieve and stored in the refrigerator for further processing. A subsample of this <2 mm fraction was taken for each soil sample and stored in separate 20 mL scintillation vials. The vials were then placed in a -20°C freezer until enzyme assays could be carried out. Sinsabaugh *et al.* (1991) recommended that freezing be avoided, and if delays were unavoidable that samples should have been stored in a refrigerator. It has been noted that freezing and subsequent thawing of samples could cause an increase in enzyme activities relative to samples that were refrigerated instead (Sinsabaugh et al., 1991). Increases in enzyme activities because of freezing could be attributable to desorption of enzymes, disruption of enzyme complexes, or stimulation of microbial activity by cell lysates (Sinsabaugh and Linkins, 1989). All samples to be analyzed for enzyme activity, however, were all treated to the same storage procedures, and thus all results would be comparable to one another. The samples were also chosen to be frozen because it would guarantee that the enzymatic activity of most of the hydrolytic and oxidative exoenzymes would remain stable for longer periods of time (Sinsabaugh et al., 1991). The objective of conducting enzyme assays on the collected samples was to measure any relative differences in activities; absolute values were not necessary. With this in mind, freezing the samples and analyzing them at a later date was more convenient than storing them in the refrigerator and having to analyze them immediately.

The activity of the above mentioned 5 hydrolytic (CBH, BG, NAG, LA, and AP) and 2 oxidative (PPO and PerO) enzymes in the bank face soil samples were measured using a

modified assay (Sinsabaugh et al., 1991; Allison and Vitousek, 2004) in order to understand microbial performance in terms of substrate utilization. The procedure recommended conducting assays under conditions of substrate saturation, which involved preparing substrate solutions with concentrations ranging from 2-5 mM. The substrates utilized in the project were: *CBH*: 2 mM pNP-cellobioside (pNP = p-nitrophenol), *BG*: 5 mM pNP-β-glucopyranoside, *NAG*: 2 mM pNP-β-N-acetylglucosaminide, *LA*: 5 mM leucine p-nitroanilide, *AP*: 5 mM pNP-phosphate, and *PPO* and *PerO*: 5 mM L-dihydroxy-phenylalanine (L-DOPA) The matrix for each of the substrate solutions consisted of a 50mM, pH 5, acetate buffer solution. The buffer was made in a large batch by dissolving 4.374 g sodium acetate trihydrate and 1.1 mL glacial acetic acid in a 1 L volumetric flask and making the solution to volume with deionized water. Each assay solution was prepared by dissolving the respective substrate in 100 mL acetate buffer: CHB: 92.7 mg, BG: 150.7 mg, NAG: 68.5 mg, LA: 125.7, AP: 185.6 mg, and one assay solution was prepared for both PPO and PerO: 98.6 mg. A final reagent, 1.0 N NaOH, was used to terminate the assay reaction for each sample and develop the color that would be analyzed on the microplate spectrophotometer. The 1.0 N NaOH solution was prepared by dissolving 40 g NaOH pellets in deionized water and making it to volume in a 1 L volumetric flask. All of the solutions used for the enzyme assay method could be used for up to ~ 2 weeks after being made if stored in the refrigerator.

After all the necessary reagents were made the soil samples were removed from the freezer and allowed to thaw to room temperature. For each sample ~2 g wet weight of soil was weighed into a blender mini-jar and its mass was recorded. After adding 60 mL of the acetate buffer to each sample the soils were homogenized by placing them on an Oster 12-Speed Blender (Model 6641) and "whipping" them for 1 minute on low speed. A stir bar was placed in

each mini-jar, and the homogenized samples were placed on a magnetic stirrer to make sure they stayed well mixed. A 0.750 mL aliquot of each sample homogenate was combined with 0.750 mL of each substrate in a 2 mL Eppendorf tube. The tips of the pipettes used to transfer the homogenate solutions were snipped off in order to prevent any clogging during the transfer of the soil slurry. For every sample, 3 analytical replicates were prepared for each of the 7 enzyme assays. Controls were included to account for the background absorbance of the homogenates and substrates. Sample (or homogenate) controls for CBH, BG, NAG, LA, AP, and PPO were made by mixing 0.750 mL of sample with 0.750 mL of acetate buffer in the 2 mL tubes, and substrate controls were made by mixing 0.750 mL of acetate buffer with 0.750 mL of substrate solution. The control solutions for PerO were prepared similarly, but instead of mixing the sample or substrate solution with acetate buffer, they were mixed with 0.750 mL of 0.3% H_2O_2. All tubes were mixed using a spinning vortex device, then placed in racks and incubated at 20°C for ~1-6 hours while constantly being shaken at 400 RPM. Incubation times for each substrate varied: CBH: 4 hours, BG: 1 hour, NAG: 3 hr, LA: 4 hr, AP: 45 minutes, and PPO and PerO: 1 hour. After incubating for the appropriate amount of time samples were placed in a table top centrifuge and spun for 2 minutes at 12,000 RPM. 150 µL of the supernatant from each sample was then pipetted into the wells of a microplate, followed by the addition of 100 µL of deionized water. To terminate the enzyme reaction, as well as to develop the color for each sample, 5 µL of 1.0 N NaOH was then added to each well of the microplate. The NaOH solution, however, was not added to the PPO and PerO assay samples. Absorbances for each of the samples were then measured using the microplate spectrophotometer. The absorbance levels for the CBH, BG, NAG, LA, and AP assays were all measured at 405 nm, while absorbances for PPO and PerO were measured at 450 nm. Thus, the supernatants were assayed colorimetrically for p-

nitrophenol and for the L-dihydroxy-phenylalanine oxidation products. Standard curves of absorbance versus concentration were generated in order to calculate an extinction coefficient needed for calculating activity levels. By measuring the absorbances of known quantities of p-nitrophenol the extinction coefficient of 3.4 was determined for the hydrolytic enzymes. An extinction coefficient of 1.8 found in the literature (Allison and Vitousek, 2004) was used for the oxidative enzymes, PPO and PerO, as we did not have any mushroom tyrosinase (the recommended standard solution) to generate a curve at the time the activities of PPO and PerO were measured. Enzyme activities were then calculated using the equation: OD/[(EC/µmol/mL)/(1.5 mL/assay)(incubation hr)(gSOM/mL sample homogenate)(0.750 mL homogenate/assay)], and reported in units of µmol substrate consumed per gram soil organic weight per hour (µmol substrate hr^{-1} g SOM^{-1}).

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